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Editors
B. C. J. G. KNIGHT A. A. MILES

Associate Editors
G. C. AINSWORTH A. T. R. MATTICK
W. B. BRIERLEY K. M. SMITH
T. GIBSON A. W. STABLEFORTH
D. D. WOODS

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Myxobacteria in Soils and Composts; their Distribution, Number and Lytic Action on Bacteria

By B. N. SINGH

Department of Soil Microbiology, Rothamsted Experimental Station, Harpenden, Herts

SUMMARY: *Myxococcus virescens*, *M. fulvus*, *Chondrococcus exiguus* and *Archangium* sp. are widely distributed in the soils of Great Britain including some treated only with artificial fertilizers. *Myxococcus virescens* and *Chondrococcus exiguus* appear to be the dominant species. The numbers of myxobacteria ranged from 2000 to 76,400/g. of soil. In an actively decomposing compost of sludge and straw the number of *Myxococcus fulvus* was found to be over 500,000/g.; *M. virescens* and *Chondrococcus exiguus* were also present, but less abundantly.

Variation of pH between 4·0 and 8·8 does not affect the growth of *Myxococcus fulvus*, *M. virescens* and *Chondrococcus exiguus*, and normal fruiting bodies were produced in the presence of suitable bacteria. *Myxococcus fulvus* and *M. virescens* do not attack filter paper.

Both Gram-positive and Gram-negative bacteria growing on solid media may be lysed by extracellular secretions of *M. virescens*, *M. fulvus* and *Chondrococcus exiguus*. Of forty-seven Gram-positive and forty-seven Gram-negative strains of bacteria tested with three species of myxobacteria, a higher percentage of Gram-negative than of Gram-positive strains was attacked. Pigmented strains of bacteria are more resistant to the lytic action of myxobacteria than the non-pigmented strains. Different species of myxobacteria and different strains of the same species differ in their lytic action on a number of species of bacteria.

The extracellular lytic substance produced by *Myxococcus virescens* passes through a cellophan membrane. A method of growing *M. fulvus* and *M. virescens* in mass cultures in liquid media to produce extracellular lytic secretions is described.

Myxobacteria were recognized as an independent group by Thaxter (1892). Although over fifty species have been described we know very little indeed about these interesting organisms apart from their morphology, and some are known only by the description of their fruiting bodies. The favourable influence of bacteria and fungi on the growth of myxobacteria was stressed by earlier workers. Pinoy (1921) was the first to observe the lysis of an unspecified *Micrococcus* by *Chondromyces crocatus* on a nutrient agar and recently Solntzeva (1939) has studied the lytic effect of *Chondromyces aurantica*, *Milittangium boletus*, *Myxococcus rubescens*, *M. virescens* and *M. filiformis* on five species of plant pathogenic bacteria. She grew the plant pathogens for 2 days on peptone + starch agar and then introduced myxobacteria into the culture. Some lysis of a few species of bacteria could be seen. When dilute suspensions of microcysts from fruiting bodies grown on rabbit-dung pellets were plated on manure extract + proteose peptone + glucose agar, Snieszko, McAllister & Hitchner (1943) observed the occasional lysis of the portions of the surface colonies of associated Gram-negative bacteria by *M. virescens*. They also showed the lysis of dead bacteria by *M. virescens* and *M. fulvus*. Beebe (1941*b*) has shown that a few species of dead bacteria are lysed by *Polyangium fuscum*, *Podangium erectum*, *Chondrococcus blasticus*, *Myxococcus fulvus* and *M. virescens*.

Although no conclusive evidence has been produced of the lysis of living bacteria by myxobacteria, it is suggested by the work of Pinoy (1921) and Solntzeva (1939). All the known higher myxobacteria so far investigated fall into two main groups: (i) those capable of effecting either partial or complete decomposition of cellulose (Krzemieniewski, 1937*b*; Imsenecki & Solntzeva, 1937; Mishustin, 1938); (ii) those capable of attacking living or dead cells of true bacteria, and possibly of other micro-organisms, and developing normally when these cells are the only food. For the first group the term 'cellulolytic' and for the second 'bacteriolytic' may be used.

Myxobacteria were considered by earlier workers to be dung organisms because they were first isolated from dung of various animals that had lain on the ground for some time. The Krzemieniewski (1926, 1937*a*) claim to have isolated a large number of species from various Polish soils by putting sterilized rabbit dung on 100–150 g. of soil in large Petri dishes. By the same method Beebe (1941*a*) isolated from American soils species belonging to Myxococcaceae and Polyangiaceae. The most common species belong to the genus *Myxococcus*. Apart from isolating myxobacteria from large quantities of soil, no effort has been made to find out how commonly they are present, as a preliminary to assessing their economic importance in soil or other substrates.

The present study of the myxobacteria followed the development of a method of counting holozoic soil Protozoa based on their differential feeding on different bacteria (Singh, 1942, 1945, 1946*a*, *b*). By this method soil and compost heaps proved to contain significant numbers of certain species of myxobacteria, lysing both Gram-positive and Gram-negative bacteria. The study of differential feeding of Protozoa on bacteria has also led to methods of isolating and purifying lytic species of myxobacteria from soils and other substrates and of counting them.

Methods of isolation and culture

The method of isolation is similar to that used for Acrasieae (Singh, 1946*c*). It consists in spreading over the surface of non-nutrient agar (1·5 % washed agar, 0·5 % NaCl) of pH 6·6–7·2, or silica jelly (for method of preparation see Singh, 1946*b*), a few loopfuls of a suitable bacterium in the form of disks or 'bacterial circles' about 1 in. in diameter. *Aerobacter*, strain 1912 (Singh, 1941), was used throughout the work described below.

The bacteria used are taken from 2–4-day cultures on nutrient agar. Several such 'bacterial circles' are made in a Petri dish. These circles are inoculated at the centre either with very small crumbs of soil or with a very small portion of a substrate such as decomposing compost, or with suspensions of soil or other substrates. The plates are incubated at 25° for 1–3 weeks and examined at intervals under a low power of a microscope. In these crude cultures large numbers of fruiting bodies of myxobacteria are usually found.

The crude cultures contain large numbers of amoeba cysts if a suitable bacterium for the development of amoebae is used. In order to discourage the development of amoebae, strains of bacteria are selected that are not suitable for the growth of amoebae, but are readily attacked by lytic myxobacteria.

It is best to use a suitable strain of Gram-negative organism, because it does not encourage the growth of the lytic actinomycetes which are abundantly present in arable soils.

The myxobacteria are purified as follows: bacterial streaks generally 1 in. long and 0·3–0·4 in. broad are made on non-nutrient agar or silica jelly and fruiting bodies are placed in contact with the bacteria at one end of the streaks. These bacterial streaks are cleared by the contaminating amoebae and by the myxobacteria within a week; some of the fruiting bodies or a portion of a swarm of myxobacteria which are formed away from the amoebae are transferred to fresh streaks. This process is repeated once or twice till a mixed culture of myxobacteria and bacteria only is obtained. The fruiting bodies formed outside the zone of bacterial streaks are now transferred to the centre of a 'bacterial circle' made with a thick suspension of a young culture in 0·9 % NaCl autoclaved at 15 lb./sq.in. for 5–10 min. The myxobacteria clear the dead 'bacterial circle' in a few days and form a large number of fruiting bodies. Some of these bodies from the edge of the circle are suspended in 0·9 % NaCl and plated in the usual way on non-nutrient agar containing a thick suspension of autoclaved bacteria, and incubated at 25°.

After a few days myxobacterial colonies develop and lyse the dead bacteria (Pl. 1, figs. 3, 4). The colonies look like those of true bacteria, smooth, flat and with entire edge; no swarming of the myxobacterial rods can be seen at the edge. The colonies are at first white, but gradually become coloured as the fruiting bodies are formed. The fruiting bodies appear in large numbers in 7–15 days, sometimes at the circumference of the colony and sometimes at the centre; finally the whole of the colony is converted into fruiting bodies.

Sub-cultures from the fruiting bodies are made on dead bacterial agar every 1–2 months, and their purity tested on nutrient agar, on which myxobacteria generally grow well but do not produce fruiting bodies. Cultures maintained on nutrient agar have to be sub-cultured every 7–10 days, as myxobacteria autolyse within 10–15 days (Snieszko *et al.* 1943). Pure cultures of species of *Myxococcus* have been maintained both on nutrient agar and on dead bacterial agar for more than 2 years. Whenever myxobacteria growing on nutrient agar are transferred to suitable strains of either dead or living bacteria on non-nutrient agar, they readily lyse the bacteria and large numbers of fruiting bodies are formed.

In addition to the media described above, sterilized fresh rabbit dung was also used to isolate myxobacteria from soils and composts but with disappointing results. The rabbit dung used was from tame rabbits and it is possible that fresh dung from wild rabbits would give better results.

Distribution and number of myxobacteria in soil and compost

Beebe (1941*a*) isolated only a few species of myxobacteria, mostly members of the Myxococcaceae, from American soils and this has also been the experience of the author with the soils of Great Britain. It is quite possible that as in the method used such a small sample of soil (0·5 g. or less) is examined only species present in large numbers have been found. Moreover, the use of bacterial strains may have completely prevented the isolation of those species which do not attack bacteria.

Sixty-nine soil samples from Hertfordshire, Berkshire, Bedfordshire, Wiltshire, Kent, Cornwall, Glamorganshire, Breconshire and Aberdeen were examined together with soils of all the classical plots of Barnfield and Broadbalk and a number of other fields at Rothamsted. The samples were taken from the top few inches of the soil. Myxobacteria were present in all the arable soils and in twenty-one of the thirty-one grassland soils. The occurrence of myxobacteria in large numbers in the classical plots of Barnfield, Broadbalk and other fields at Rothamsted, where no farmyard manure has been added, in some cases for 100 years or more, disproves the earlier belief that myxobacteria are dung organisms. Their common occurrence in dung of various animals that has been lying on the ground for several days suggests that they find suitable nutritive material in those substrates.

No correlation between the pH of the soils and the distribution of species could be found in soils ranging from pH 4·0–8·0, although a correlation with soil pH was claimed by the Krzemieniewski (1926).

On several occasions a few species belonging to groups other than the Myxococcaceae were present both in bacterial and in rabbit-dung cultures used to isolate species of myxobacteria. They did not grow in culture; and, until this is done, it does not appear justifiable to name them from the fruiting bodies.

Myxococcus virescens, *M. fulvus*, *Chondrococcus exiguus* and *Archangium* sp. are the species which have so far been isolated and cultured from soils very widely scattered in Great Britain (Pls. 1 and 2, figs. 1–8). *Myxococcus virescens*, *Chondrococcus exiguus* and *Archangium* sp. were very commonly found in soil, but in the few samples of decomposing compost of sludge and straw examined *Myxococcus fulvus* occurred in much larger numbers than the other three species.

When nineteen strains of bacteria consisting of both Gram-positive and Gram-negative were selected at random, and used to isolate species of myxobacteria from the farmyard manured soils of Barnfield and Broadbalk plots at Rothamsted only the four species mentioned above could be isolated.

A few counts have been made by the dilution method of Singh (1946*a*, *b*) on Barnfield farmyard manured soil and on a decomposing compost heap of sludge and straw set up at Rothamsted farm. The identification of myxobacteria at various dilutions was by the fruiting bodies. Table 1 shows three counts made on different dates from Barnfield farmyard manured soil, using *Aerobacter* on non-nutrient agar substrate. In every case *Myxococcus virescens* was dominant.

Two counts were made from an actively decomposing compost of sludge and straw in March 1945, from samples taken at 3 and 6 in. depth, on *Aerobacter* in silica jelly. The numbers of myxobacteria were found to be over 500,000/g. of compost from the sample taken at 3 in. depth and not more than 1000 at 6 in. depth, where the temperatures were respectively 30° and 36°. It is possible that anaerobic conditions or the increase in temperature, or both, may account for this big difference in numbers at different depths.

Myxococcus fulvus, *M. virescens* and *Chondrococcus exiguus* were isolated and purified from these crude cultures. *Myxococcus fulvus* alone was present in the very high dilutions. The result of this count in compost suggests that a detailed study of this group of organisms in various types of composts (hot and cold) might be interesting, especially as nothing is known about the role of myxobacteria in soil or in decomposing compost heaps.

Table 1. *Counts of lytic myxobacteria in an arable soil*

Barnfield farmyard manured soil	Number of myxobacteria per g.
First count	76,399
Second count	6,000
Third count	2,000

Effect of pH *on the growth of myxobacteria*

The effect of pH on the growth of *M. virescens*, *M. fulvus* and *Chondrococcus exiguus* was tested as follows. Non-nutrient agar was adjusted to pH 4·0, 4·6, 5·0, 5·5, 6·0, 6·6, 7·0, 7·5, 8·1 and 8·8. The agar was poured into Petri dishes, streaked with *Aerobacter* sp., and inoculated with fruiting bodies of myxobacteria. All species grew well and produced normal fruiting bodies at all the pH values.

Growth of Myxococcus virescens *and* M. fulvus *on filter paper in the presence and absence of bacterial suspensions*

When fruiting bodies from pure cultures of *M. virescens* and *M. fulvus* were transferred to strips of filter paper dipped in mineral salt solution (0·1 % K_2HPO_4, 0·05 % KCl, 0·05 % $MgSO_4$, 0·1 % $NaNO_3$, trace of $FeSO_4$) in test-tubes and incubated at 25°, no apparent growth or decomposition of the filter paper took place up to 2 months. When a suspension of dead bacteria was added to the solution both the *M. virescens* and *M. fulvus* grew well and produced large numbers of fruiting bodies. The fruiting bodies were mostly formed on the glass, and some on the filter paper above the surface of the liquid. Thick bacterial suspensions were usually cleared within 2 weeks. Cultures maintained for more than 2 months on strips of filter paper in suspensions of dead bacteria did not show any sign of decomposing the filter paper.

Some workers have used a dilution method of counting *Sporocytophaga myxococcoides* (*Spirocheta cytophaga*; see Stanier, 1942*a*), noting the yellow colour developing on filter paper, which is decomposed. When *Myxococcus virescens* is put on filter paper in the presence of a bacterial suspension it produces a colour like that of *Sporocytophaga myxococcoides* but does not decompose the paper. The method of counting *S. myxococcoides* is very unsatisfactory since there is no guarantee that both the yellow colour and the decomposition of filter paper is caused by *S. myxococcoides*. It is possible that the yellow colour is due to *Myxococcus virescens* and the decomposition of the filter paper is due to cellulose-decomposing organisms other than *Sporocytophaga myxococcoides*. It is difficult to be certain of the presence of *S. myxococcoides* by microscopic examination since the vegetative cells both of it and of *Myxococcus virescens* are alike. The only way to make sure of the presence of *Sporocytophaga myxococcoides* is to isolate this organism from every dilution or at least from a few higher dilutions where the yellow colour is associated with the decomposition of filter paper.

Lysis of living bacterial strains by species of Myxococcaceae on solid media

Myxococcus virescens, *M. fulvus* and *Chondrococcus exiguus* isolated from a decomposing compost heap, and a strain of *Myxococcus virescens* isolated from a Rothamsted soil, were tested for bacteriolytic activity as follows. Three widely separated streaks of a 2–4-day culture of a bacterium on nutrient agar were made on non-nutrient agar. Two streaks were inoculated at one end with fruiting bodies of one of the myxobacteria growing with living *Aerobacter* sp. and the third was left as control. The cultures were incubated at 25° and examined at 5 and 10 days. Of the bacteria tested by this method some were readily or slowly but completely lysed, others showed very slight partial lysis and the rest were not lysed within a period of 10–12 days. Table 2 shows the lytic action of the three species of Myxococcaceae on sixty-two strains of various bacteria. The three species differ in their reactions towards the same strain of bacteria in a number of cases. Against *Rhizobium* and plant pathogens they behave, on the whole, in a similar manner, nearly all the plant pathogens being readily attacked whereas the strains of *Rhizobium* seem to be resistant. Two strains of *Myxococcus virescens*, one isolated from a compost heap and the other from a soil, differed in their lytic action towards eight of the fifty-six strains of bacteria. Some strains of bacteria, not lysed by the soil strain of *M. virescens*, were lysed by the strain isolated from compost and vice versa. Pigmented strains seem to be more resistant than the non-pigmented (see Table 3). In Table 4 the lytic action towards Gram-positive and Gram-negative organisms is shown. Though both Gram-positive and Gram-negative bacteria are attacked, more Gram-negative than Gram-positive strains are attacked except by *M. virescens*.

Table 2. *The lysis of bacterial strains by myxobacteria*

CL = Completely lysed either readily or slowly; PL = very slight partial lysis; NL = not lysed.

	M. virescens			*M. fulvus*			*C. exiguus*			Total strains tested
	CL	PL	NL	CL	PL	NL	CL	PL	NL	
Common soil bacteria	9	1	1	10	—	1	8	3	—	11
Rare soil bacteria	16	10	3	8	12	9	5	10	14	29
Plant pathogens*	9	—	1	9	—	1	9	—	1	10
Strains of *Rhizobium*	3	6	3	4	4	4	3	6	3	12

* The strains (4752, 5945, 1989, 5944, 5942, 385, 5943, 387, 5241 and 1997) of plant pathogens were obtained from the National Collection of Type Cultures, Lister Institute. See Singh (1942) for the names of the plant pathogens used.

Nineteen strains of soil bacteria were tested to see whether the Myxococcaceae actually attack living bacteria on solid media or only lyse cells that are dead. After 10 days' incubation, slopes of nutrient agar were streaked with samples from the control cultures, and material from the lysed areas on the plates supplied with myxobacteria was similarly subcultured. Growth of the samples from the control streaks was profuse in 24 hr. at 25° while those from areas lysed by *M. fulvus* in every case grew only slightly within 2–4 days. Subcultures from the lysed areas produced by *M. virescens* on sixteen strains of bacteria failed to grow in seven cases, even after 10 days' incubation, whereas nine gave positive results within 2–5 days. Cultures from areas lysed by *Chondrococcus exiguus* were positive with nine strains of bacteria tested and negative with two.

Table 3. *The lysis of pigmented and non-pigmented strains of bacteria by myxobacteria.* (*Strains of* Rhizobium *have been excluded*)

CL = Completely lysed either readily or slowly; PL = very slight partial lysis; NL = not lysed.

	No. of strains tested	*M. virescens*			*M. fulvus*			*C. exiguus*		
		% CL	% PL	% NL	% CL	% PL	% NL	% CL	% PL	% NL
Pigmented bacteria	41	63·4	19·5	17	41·4	34·2	24·4	36·5	24·4	39·1
Non-pigmented bacteria	52	75	17·3	7·6	67·3	19·2	13·4	59·5	25	15·5

Table 4. *The lysis of Gram-positive and Gram-negative bacteria by myxobacteria.* (*Strains of* Rhizobium *have been excluded*)

CL = Completely lysed either readily or slowly; PL = very slight partial lysis; NL = not lysed.

	No. of strains tested	*M. virescens*			*M. fulvus*			*C. exiguus*		
		% CL	% PL	% NL	% CL	% PL	% NL	% CL	% PL	% NL
Gram-positive	47	68	21·3	10·6	38·3	34	27·6	31·9	38·3	29·8
Gram-negative	47	74·4	14·9	10·6	74·4	17	8·5	68	10·7	21·2

In a second experiment, asparagine agar (Thornton, 1922) was selected as a substrate allowing slow bacterial growth. A loopful of *Aerobacter* sp. from a 36-hr. culture was streaked on asparagine agar in a Petri dish and inoculated with fruiting bodies of *Myxococcus virescens*. Roughly the same amount of bacteria was streaked as a control. Pl. 2, figs. 9 and 10, show the results obtained. It is clear that in 4 days *M. virescens* had completely lysed the growing cells of the bacteria and large numbers of fruiting bodies were produced. When a pure culture of the fruiting bodies of *M. virescens* is inoculated on asparagine agar, it grows slowly but does not produce fruiting bodies. The lysis of living cells of *Aerobacter* sp. on asparagine agar by *M. fulvus* and *Chondrococcus exiguus* is not so spectacular as that by *Myxococcus virescens*; it is not complete until after 5–7 days. *M. fulvus* and *Chondrococcus exiguus* grow well on this medium but they too do not produce fruiting bodies in the absence of suitable bacteria.

The numbers of bacteria viable in the control and the lysed area were determined as follows. A loopful of bacteria from the edge and from the centre of the control streak on the 4th day (see Pl. 2, figs. 9, 10) were each suspended in 10 ml. of 0·9 % NaCl solution and a total count made in a haemocytometer. The number of viable bacteria was determined by plating dilutions of the suspensions. Similarly, one loopful of myxobacterial growth from the lysed area was suspended in 10 ml. of NaCl solution; the suspension was diluted one-hundred-fold and plated for counting. The control bacterial growth contained 74–85 % viable cells. In areas lysed by *Myxococcus fulvus* very few living cells of *Aerobacter* sp. were found and none in areas lysed by *Myxococcus virescens* or *Chondrococcus exiguus*. These data show that the myxobacteria kill the living bacteria and do not merely lyse dead bacterial cells.

It has been repeatedly found that the pigmentation of both *Myxococcus virescens* and *M. fulvus* is influenced by the bacterial strain supplied as food. The fruiting bodies of *M. virescens* were found to be brown, brick red, whitish, yellowish red, etc., according to the strain of bacterium used; the pigmentation of *M. fulvus* varied in the same way but less markedly. Similar variations in pigmentation were noticed by Snieszko *et al.* (1943). The size of the fruiting bodies is also influenced by the type of bacteria used. Different shapes of fruiting bodies are produced even in the same culture (see Stanier, 1942*b*, for the explanation of this phenomenon). In the past, species have been based on the size, shape and pigmentation of fruiting bodies. In the light of the above results, it must be emphasized that the characters can only be used for diagnostic purposes when the organisms have been grown under strictly controlled conditions.

Passage of the lytic substance of M. virescens *through cellophan*

The experiment was arranged as follows. In the centre of non-nutrient agar plates a few loopfuls of *Aerobacter* sp. from a 2-day culture on nutrient agar was spread out as a thin layer; a sterilized cellophan membrane was laid over this and on the upper surface microcysts from a pure culture of *Myxococcus virescens* were streaked in the centre of the plate. Within 2 weeks it could be seen that a very large proportion of the bacteria beneath the cellophan had been lysed, and large numbers of fruiting bodies were formed on the top of the membrane. No decomposition of cellophan occurred within a month. From this experiment it is clear that the bacteriolytic substance produced by *M. virescens* passes through cellophan, and the products of lysis diffuse through cellophan and supply nutrient material for the myxobacteria.

Method of growing M. virescens *and* M. fulvus *in mass liquid culture to produce the lytic substance*

Since it was observed that both *M. virescens* and *M. fulvus* produced fruiting bodies only above the surface of liquid media and that in a decomposing compost heap at a depth of 6 in. myxobacteria were present only in very small numbers, it was thought that they needed air for luxuriant growth. The organisms were grown in liquid media as follows: 5–7 days' growth of *Aerobacter* sp. from nutrient agar slope was gently scraped off in 0·9 % NaCl solution and collected in a flask. 750 ml. flasks containing 75 ml. of 0·9 % NaCl solution and 25 ml. of the thick suspension of bacteria were autoclaved at 15 lb./sq.in.

for 10 min. Myxobacteria growing in pure culture, either on nutrient agar or on dead bacterial suspension agar, were inoculated into these flasks and incubated at 25°.

Within 2 weeks the bacterial suspensions were completely cleared, and good growth of the myxobacterial colonies on the glass below the shallow layer of liquid could be seen within a week. These colonies were sometimes very large and gelatinous; they were white at first and later coloured as the fruiting bodies formed. Within 2–3 weeks very large numbers of fruiting bodies were formed both on the bottom of the flask below the liquid and on the glass above the liquid surface. An improved development of fruiting bodies by *Myxococcus virescens* and *M. fulvus* on the bottom could be induced by covering the bottom of the flask with a layer of plain agar.

When suspensions of living *Aerobacter* sp. were used in flasks to grow myxobacteria, there was very little lysis of the bacteria up to a period of 2 weeks, and little growth of myxobacteria.

DISCUSSION

Although great attention has been paid in recent years to isolating antibiotics from various groups of micro-organisms, myxobacteria have so far received none. As some species of the Myxococcaceae lyse living bacteria, at least on solid media, a detailed study of the organisms belonging to the myxobacteria may be profitable in discovering new antibiotics active especially against Gram-negative organisms. The property of lysing Gram-negative organisms such as *Pseudomonas fluorescens* and *Bacterium coli*, by extracellular secretions, has rarely been found in other groups of micro-organisms. Moreover the fact that amoebae grow luxuriantly and produce cysts in crude cultures that also contain actively growing myxococci, suggests that the extracellular secretions of the myxococci do not affect amoebae, and hence may be non-toxic to animal tissues.

This work was made possible by a grant from the Agricultural Research Council, to whom the author's thanks are due.

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EXPLANATION OF PLATES

Plate 1

Fig. 1. Vegetative cells of *Myxococcus virescens* fixed by Carnoy and stained by iron alum haematoxylin. × 1000.

Fig. 2. Microcysts of *M. virescens* fixed by Carnoy and stained by iron alum haematoxylin. They are spherical or oval and never cup-shaped. × 1000.

Fig. 3. Colonies of *Chondrococcus exiguus* developing on a suspension of dead *Aerobacter* sp. in agar. Five-days-old culture. Clearing of the bacterial cells round the colonies can be seen. × 4.

Fig. 4. Colonies of *C. exiguus* similarly produced. Some colonies show the beginning of fruiting bodies formation as granular patches in the centre. × 4.

Fig. 5. Fruiting bodies of *Myxococcus virescens* formed on sterilized rabbit dung. × 42.

Fig. 6. Fruiting bodies of *M. fulvus* produced on non-nutrient agar with *Aerobacter* sp. as food supply. × 42.

Plate 2

Fig. 7. Fruiting bodies of *Chondrococcus exiguus* produced on non-nutrient agar with *Aerobacter* sp. as food supply. Photographed by reflected light. × 60.

Fig. 8. The same as Fig. 7. Photographed by transmitted light.

Fig. 9. Growth of *Aerobacter* sp. on asparagine agar. Four-days-old culture (control). × 6.

Fig. 10. Culture of the same age as in Fig. 9 inoculated with *Myxococcus virescens* showing large numbers of fruiting bodies and the complete lysis of *Aerobacter* sp. × 6.

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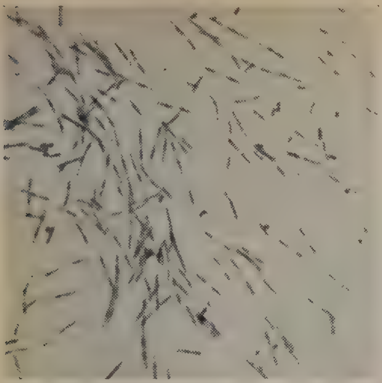


Fig. 1

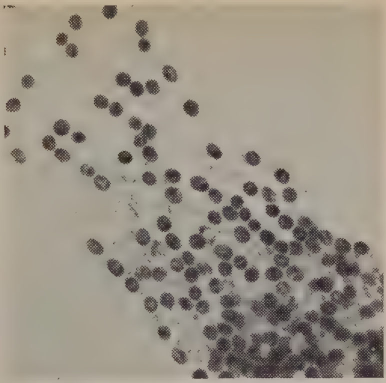
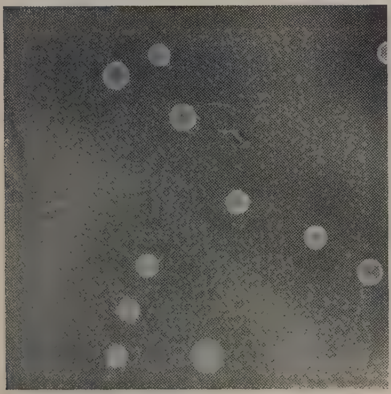


Fig. 2



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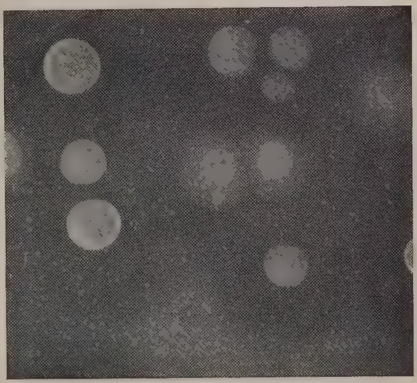

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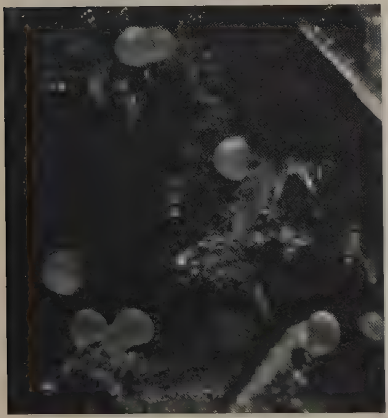

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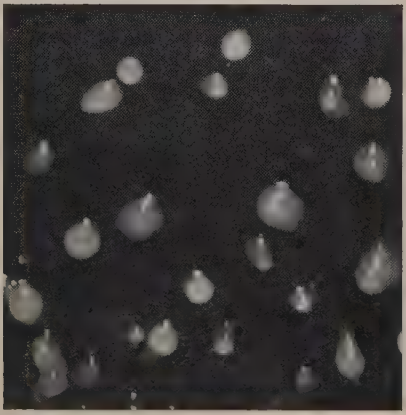


Fig. 6

B. N. Singh—Myxobacteria in soils. Plate 1

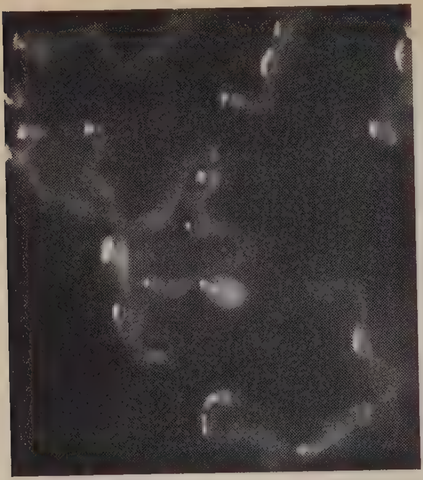


Fig. 7

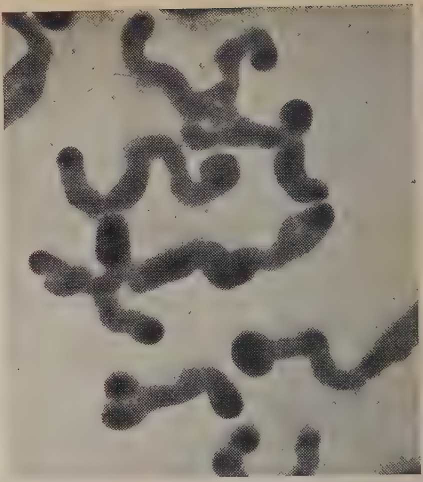


Fig. 8

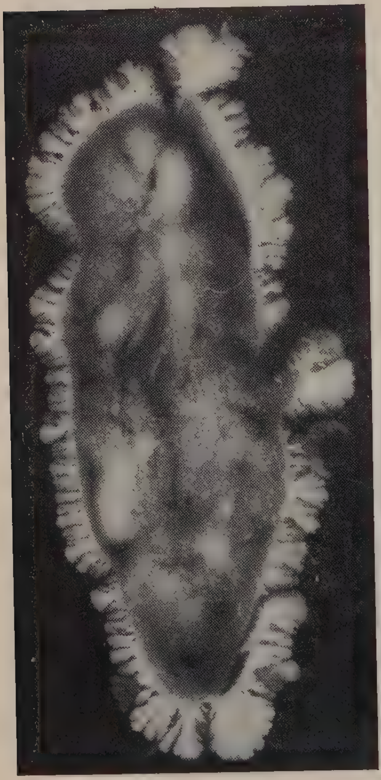


Fig. 9

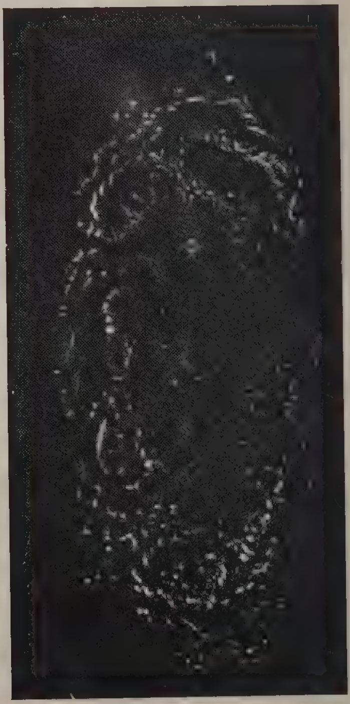


Fig. 10

B. N. Singh—Myxobacteria in soils. Plate 2

Studies on Soil Acrasieae

1. Distribution of species of *Dictyostelium* in soils of Great Britain and the effect of bacteria on their development

By B. N. SINGH

Department of Soil Microbiology, Rothamsted Experimental Station, Harpenden, Herts

SUMMARY: A method of isolating species of Dictyosteliaceae from soil and other substrates is described.

Of ninety-three different strains of bacteria tested as food for *Dictyostelium giganteum* and *D. mucoroides*, some were readily or slowly but completely eaten, others were partly eaten and the rest were inedible. No correlation between the edibility of the bacteria and the formation of normal fruiting bodies could be found. More Gram-negative than Gram-positive strains were edible, and non-pigmented bacteria proved more suitable than pigmented bacteria for the normal development of *Dictyostelium* spp.

Values of pH between 4·1 and 8·9 had no effect on the abundance or on the types of fruiting bodies produced in either *D. giganteum* or *D. mucoroides* when suitable strains of bacteria were supplied as food to the myxamoebae on non-nutrient agar.

The maximum dimensions and the form of sorocarps and occasionally the colour of the sori were influenced by the type of bacteria used as food supplies. The influence of the bacterial food supply on the classification of Acrasieae is discussed and one new species (*D. giganteum*) is described.

Species of *Dictyostelium* are frequently present in arable soils in Great Britain. The common occurrence of *Dictyostelium* spp. in soils, which have been unmanured or treated with artificial fertilizers only for 100 years or more, disproves the belief that *Dictyostelium* spp. are dung organisms.

The amoeboid protists included in the Acrasieae apparently occupy a position near the divergence of the plant and animal kingdoms. The vegetative stage consists of myxamoebae which feed and reproduce like true amoebae, but under suitable conditions of culture produce fruiting structures of definite and consistent form. The Acrasieae have been considered by botanists as plants and by protozoologists as Protozoa.

The genera *Dictyostelium* and *Polysphondylium* were created by Brefeld in 1869 and 1884 respectively and species belonging to these genera have since been described by various workers (cf. review by Raper, 1940*a*). It was not, however, until recently that Raper (1937, 1939, 1940*a*,*b*) threw considerable light on the cultural characters and food requirements of the myxamoebae of *Dictyostelium discoideum*; his studies have led to the development of a better way of cultivating, classifying, and studying organisms included in Acrasieae.

The occurrence of *Dictyostelium* spp. in Polish and American soils has been recorded by Krzemieniewski (1927) and by Raper & Thom (1932). *Polyspondylium* spp. have been rarely seen in soils. In 1944 Raper stated in a personal communication that he had isolated *Dictyostelium* spp. from soils from Australia, India, Mexico, Brazil, Cuba and numerous stations throughout the United States.

The study of differential feeding by soil protozoa (especially amoebae) carried out by the writer on a large number of 'miscellaneous' strains of bacteria (Singh, 1941, 1942, 1945, 1946*a*,*b*) led to improvements in isolating and studying members of the Acrasieae. A method of counting holozoic protozoa (Singh, 1946*a*,*b*) has been developed which is based on the differential feeding of amoebae. In this method a pure culture of a suitable bacterial species or a mixture of a few species is given as food for the development of protozoa on a substrate of non-nutrient agar or silica jelly. The use of this method revealed the common occurrence of *Dictyostelium* spp. in arable soils.

Method of isolation and culture of Dictyostelium *spp.*

The method of isolation has been described (Singh, 1946*c*). One or two loopfuls of a suitable bacterium growing usually on nutrient agar (2–7-day culture) is spread over the surface of non-nutrient agar (1·5 % washed agar in 0·5 % NaCl; pH 6·5–6·8) in the form of a disk or 'bacterial circle' of about an inch in diameter. In this way the growth of micro-organisms except those that feed on bacteria is checked. Several such circles are made in each Petri dish. These cultures are then inoculated at the centres either with very small crumbs of soil, diluted soil suspension, or with small portions of some other substrate and the plates then incubated at 21–23° for 10–15 days and examined at intervals.

The myxamoebae feed on the bacteria, reproduce actively and then form a pseudoplasmodium which gives rise to a fruiting body. All these stages can be seen in the crude cultures by examining the plates at frequent intervals. The crude cultures usually contain large numbers of true amoebae, both active and cystic, fruiting bodies of myxobacteria (Singh, 1947), fruiting bodies and myxamoebae of *Dictyostelium* spp. and other micro-organisms which feed on bacteria. The myxamoebae of the Dictyosteliaceae are indistinguishable from small true amoebae. In order to encourage the growth of the Dictyosteliaceae, strains of bacteria are selected which are readily eaten by the myxamoebae and lead to the production of normal fruiting bodies but which are not suitable as food for the active development of the amoebae. It is preferable to use Gram-negative strains of bacteria, because these do not encourage the development of lytic actinomycetes which are abundantly present in arable soils.

From the crude cultures species of Dictyosteliaceae are isolated in 'pure mixed' cultures as follows. A spore mass or sorus raised well above the surface of the agar and free from fungal growth is gently punctured by a fine sterilized needle and the spores transferred to freshly prepared 'bacterial circles' on non-nutrient agar plates. Within a few days large numbers of fruiting bodies are formed. By repeating this process a few times 'pure mixed' cultures of *Dictyostelium* spp. are obtained living on one species of bacteria. Sub-cultures are made every 1–2 months in order to maintain stock cultures.

Aerobacter strain 1912 (Singh, 1941) was extensively used in the beginning of the work to isolate species of *Dictyostelium* from various soils, and now this strain is mainly used to maintain stock cultures.

Influence of bacterial food on the growth of two species of Dictyostelium

The role of bacteria in the nutrition of myxamoebae was not understood by the majority of the earlier workers (see Raper, 1937), although a few of them grew *D. mucoroides* in association with several species of bacteria on solid media containing nutrients. Raper (1937) and Raper & Smith (1939) carried out detailed work to test the effects of more than thirty saprophytic and twenty-two strains of pathogenic bacteria (animal and plant pathogens) on the growth of *D. discoideum.* Hay infusion agar of constant composition was mostly used as a substrate. The bacteria to be tested were grown for 2–3 days on hay infusion agar and then spores of *D. discoideum* were inoculated on to the same cultures. The comparative growth of *D. discoideum* in association with different bacteria was determined after 6–8 days by counting the number of large, medium, and small sorocarps. Raper (1937) found that, on the whole, better growth occurred with Gram-negative than with Gram-positive species. *D. discoideum* also grew well and consumed the colonies of nearly all the associated pathogenic bacteria grown on hay infusion agar. *D. mucoroides*, *D. purpureum*, and *Polysphondylium violaceum* grew well on several of the pathogenic bacteria tested (Raper & Smith, 1939).

Many kinds of infusions and nutrients have been incorporated in agar to grow Acrasieae. In the long experience of the writer it has been found that agar containing nutrients is neither satisfactory for testing the growth of Protozoa on different bacteria nor for counting their numbers in soil or other substrates (Singh, 1941, 1942, 1945, 1946*a*, *b*). Nutrient media encourage the development of inedible organisms introduced with the inoculum, and may even encourage the growth of micro-organisms that produce substances having a detrimental effect on the growth of protozoa (Singh, 1945) and other protists. As myxamoebae, like true soil amoebae, feed selectively on bacteria, it is necessary that the cultural conditions should be properly controlled in order to test the effects of bacteria on the growth of the species of *Dictyostelium.* The writer considers it essential to use a non-nutrient substrate to isolate, to study the effects of bacterial food supplies, and to do quantitative work on those protists which can develop normally on bacteria as the exclusive source of food supply, if consistent and reliable results are to be obtained.

In order to study the effects of bacterial strains on *Dictyostelium*, 'bacterial circles' on non-nutrient agar were inoculated at the centres with spores of *Dictyostelium* spp. Cultures of bacteria of the same age (2–5 days) from nutrient agar slopes were always used to control the results. The plates were incubated at 21–23° for 7–10 days. The main object has been to see what strains of bacteria are eaten by the myxamoebae and what effects these strains have on the production of fruiting bodies. The use of non-nutrient agar checked the multiplication of bacteria and no detectable change in the pH of the agar was observed where 'bacterial circles' were made. It can be assumed that the varying effects of different bacteria on the growth of myxamoebae and on the formation of fruiting bodies described in this paper are due to differences between the bacterial strains used.

Table 1 shows the effects of ninety-three strains of bacteria on two species of *Dictyostelium*, *D. mucoroides* and *D. giganteum* a species proposed later as new. The bacteria used comprise eleven common, twenty-seven rare strains from soil, ten strains of plant pathogens (4752, 5945, 1989, 5944, 5942, 385, 5943, 387, 5241 and 1997; cf. Singh, 1942, for the names of these strains), two strains of *Rhizobium*, and forty-three strains of 'miscellaneous' bacteria mostly isolated from soil. The bacteria tested fall into three groups. Some of the bacterial strains were completely eaten, either readily or slowly, others were only partly eaten and the rest were not eaten by the myxamoebae even when they were the only food supply. Several strains of bacteria were either completely or partly eaten by the myxamoebae but induced the production of fruiting bodies which were abnormal in character. There seems to be no correlation between edibility and the production of normal fruiting bodies. The behaviour of the two species of *Dictyostelium* towards the different bacterial strains is similar except for a few strains. All the plant pathogenic bacteria were completely eaten and normal fruiting bodies were formed except in the case of strain 5241. This strain though eaten by the myxamoebae led to their producing abnormal fruiting bodies. Table 2 shows the edibility of Gram-positive and Gram-negative strains of bacteria for two species of *Dictyostelium*. Gram-negative strains were somewhat more frequently eaten than the Gram-positive, in accord with the findings of Raper (1937). In Table 3 the relation between pigmentation and edibility of bacterial strains is shown. The two species of *Dictyostelium* had a marked preference for non-pigmented strains. Red, violet, or green strains were least suitable for the growth of *Dictyostelium* spp. although unlike soil amoebae these organisms can feed on some such strains.

Table 1. *Effects of bacterial food supply on the formation of fruiting bodies* (*FB*) *of two species of* Dictyostelium

	Completely eaten		Partly eaten		Not eaten or slightly eaten. Few or no FB, mostly abnormal	Total bacterial strains tested
Species	Normal FB	Abnormal FB	Normal FB	Abnormal FB		
D. giganteum	49	6	17	9	12	93
D. mucoroides	55	5	16	9	8	93

Influence of pH *on the growth of* D. giganteum *and* D. mucoroides

Raper (1939) deals with the effects of different nutrients incorporated in agar on the growth of *D. discoideum* with a few bacterial associates. He found that 1·2 % peptone and a carbohydrate that could be fermented by the associated bacteria, provided a suitable substrate for the growth of the host bacteria and for the subsequent normal development of *D. discoideum*. In the absence of a fermentable carbohydrate the colonies of host bacteria became alkaline in reaction, owing to the accumulation of ammonia liberated from the peptone, and toxic to *D. discoideum*. The colonies of the same bacteria in the presence of a fermentable carbohydrate remained neutral or slightly acid because of the neutralization of the ammonia by acid products simultaneously formed from the carbohydrate, and these colonies were non-toxic to *D. discoideum*. Raper also claimed that the development of *D. discoideum* was governed by the pH of the bacterial colonies. The optimum reaction was pH 6·0–6·5 while excellent growth and normal fruiting bodies were formed in bacterial colonies between pH 5·0 and 7·0. Outside this range the growth of *D. discoideum* progressively decreased and the development became abnormal. All growth ceased at pH 8·5–8·6 and at pH 4·0–4·2.

Table 2. *Edibility of Gram-positive and Gram-negative strains of bacteria for two species of* Dictyostelium

	Completely eaten		Partly eaten		Not eaten or slightly eaten. Few or no FB, mostly abnormal	Total bacterial strains tested
Species	Normal FB	Abnormal FB	Normal FB	Abnormal FB		
D. giganteum	20	1	11	6	8	Gram-positive 46
D. giganteum	29	5	6	3	4	Gram-negative 47
D. mucoroides	24	1	11	5	5	Gram-positive 46
D. mucoroides	30	5	5	4	3	Gram-negative 47

FB = fruiting bodies.

Table 3. *Edibility of non-pigmented and pigmented strains of bacteria for two species of* Dictyostelium

	Completely eaten		Partly eaten		Not eaten or slightly eaten. Few or no FB, mostly abnormal	Total bacterial strains tested
Species	Normal FB	Abnormal FB	Normal FB	Abnormal FB		
D. giganteum	32	2	5	6	6	Non-pigmented 51
D. giganteum	16	4	12	4	6	Pigmented 42
D. mucoroides	34	2	8	4	3	Non-pigmented 51
D. mucoroides	20	3	10	5	4	Pigmented 42

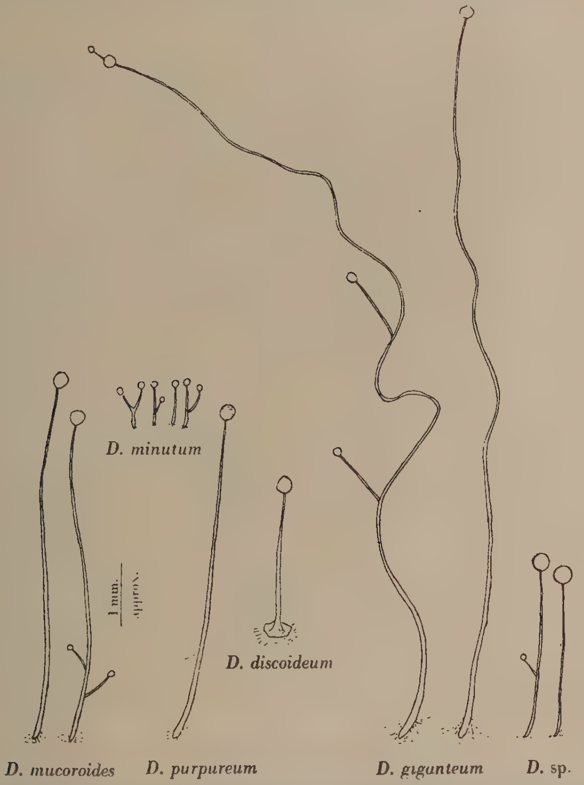
FB = fruiting bodies.

A simple experiment was devised to test the effects of pH on the growth of *D. giganteum* and *D. mucoroides* when fed with suitable bacteria. 'Bacterial circles' of *Bacterium coli*, *Pseudomonas fluorescens* and *Aerobacter* strain 1912 were made on non-nutrient agar adjusted to pH 4·1, 4·4, 5·4, 5·7, 6·0, 6·6, 7·0, 7·5, 8·2 and 8·9. Spores of *Dictyostelium* spp. were inoculated in the centre of some of these cultures while others were left uninoculated as controls. The plates were incubated at 21–23°. Within 3–5 days large numbers of normal fruiting bodies were produced and the bacterial cultures were completely eaten by the myxamoebae. At the end of 5 days bacteria from the control 'bacterial circles' were scraped off and a drop of indicator was added to the agar in this area and another drop to the agar away from the culture. No change could be detected in the pH of the agar in the area of the bacterial culture compared with agar away from it. Similarly no change in the pH of the agar could be found where slime moulds had grown with bacterial associates for 5 days. Thus it is reasonable to assume that pH values between 4·1 and 8·9 have no effect in determining the abundance or the types of fruiting bodies produced, either in *D. giganteum* or *D. mucoroides*. It is quite possible that the contrary results reported by Raper (1939) in the case of *D. discoideum* may be due not to the pH of the medium but rather to the metabolic products produced by the bacterial associates growing on the nutrients in the agar.

Influence of bacterial food supplies on the classification of the Acrasieae

The study of the effects of bacteria on the formation of fruiting bodies in Dictyosteliaceae and other groups of the Acrasieae is important in view of the confusion existing in the classification. Species of Dictyosteliaceae are generally classified by one or more of the following characters: (1) the colour of the spore mass or sorus, (2) the form of the fruiting bodies or sorocarps, and (3) the maximum dimensions of the sorocarps.

It has been found that some of the coloured strains of bacteria occasionally lead to the production of a few sori of red or yellowish colour. When the spores from these sori are cultured on the same or different suitable bacteria the sori produced are colourless. The fruiting bodies formed with some of the bacterial associates assume various abnormal shapes and forms. They may be either clumped together or one stalk may possess several sori; some of the fruiting bodies do not possess any stalks (Pl. 1, figs. 3, 5, 6, 7) and look like the fruiting structures of *Guttulinopsis* Olive and *Guttulina* Cienk. (Raper, 1940*a*). As the genera *Guttulinopsis* and *Guttulina* were described before the cultural characters of the amoeboid protists included in the Acrasieae were properly understood, it will be necessary to study these genera, if they are rediscovered, under controlled cultural conditions before they can be considered valid. The dimensions of the sorocarps also vary to such an extent that it is impossible to maintain species based on this character alone unless the organism has been grown under strictly controlled conditions and with a range of suitable bacterial foods. *Dictyostelium giganteum* (Text-fig. 1 and Pl. 1, fig. 1) produces very long creeping stalks when grown on suitable bacterial associates, but on other bacterial associates the sorocarps are very short indeed and abnormal in character. Similar changes in the dimensions of the sorocarps have been found in *D. mucoroides*. Several species of the genus *Dictyostelium*: *D. sphaerocephalum*, *D. brevicule*, *D. roseum*, *D. lacteum* and *D. aureum* (Raper, 1940*a*) should only be considered valid if their diagnostic characters can be reproduced under controlled cultural conditions. So far only four easily identifiable species have been adequately studied (*D. mucoroides* Brefeld, 1869; *D. purpureum* Olive, 1901; *D. discoideum* Raper, 1935; and *D. minutum* Raper, 1941). Another species, *D. giganteum*, is now added to this list.



Text-fig. 1. Diagrammatic representation of the identifiable species of *Dictyostelium*. The figures are reproduced from Raper (1940*a*) excepting *D. giganteum* and *Dictyostelium* sp. *D. mucoroides* characterized by milky white sorus and long sorophore sometimes bearing one or more lateral branches; *D. purpureum* characterized by deep purple sori and unbranched sorophores; *D. discoideum* characterized by basal disk and erect tapering sorophores; *D. minutum* characterized by diminutive sorophores branched and unbranched; *D. giganteum* characterized by very long creeping sorophore sometimes bearing one or more lateral branches and small milky white sorus; *Dictyostelium* sp. characterized by short sorophore occasionally bearing one or two branches and with milky white sorus.

Dictyostelium giganteum sp.nov.

In agaro non nutriente cultum cum Bacterio coli, Pseudomonate fluorescente *vel* Aerobactro 1912 *ad* 21–23°*: Myxamoebis varia magnitudine* 10–18 × 7–12 μ*; sorocarpis repentibus, plerumque* 17–25 *mm. longis, interdum usque ad* 30 *mm., saepe uno vel pluribus ramis lateralibus; sorophoris albis parte terminali flexuosis; soris rotundatis, interdum ovalibus, plerumque* 150–300 μ *in diam., maioribus et minoribus frequentibus, lacteo-albis; Sporis ellipticis, varia magnitudine*

5–10 × 2–3·5 μ. *Ex cumulo putrescentis straminis ad fundum, Rothamsted, Harpenden, Herts. Sept.* **1943.**

"Cultivated upon non-nutrient agar with *Bacterium coli*, *Pseudomonas fluorescens*, or *Aerobacter* strain 1912 at 21–23°: Myxamoebae of variable size 10–18 × 7–12 μ; sorocarps creeping, usually 17–25 mm. long, occasionally up to 30 mm., frequently with one or more lateral branches; sorophores white, terminal region flexous; sori rounded, occasionally oval, commonly 150–300 μ in diameter with larger and smaller specimens frequent, milk white; spores elliptical, size variable 5–10 × 2–3·5 μ. Isolated from an actively decomposing compost heap of straw and sludge, Rothamsted farm, Harpenden, Herts, September, 1943.'

The detailed life cycle of this species has not yet been ascertained. The important stages in its life cycle are similar to those of *D. mucoroides*. *D. giganteum* is distinguished from other species by long creeping stalks and small sori (see Pl. 1, fig. 1, and Text-fig. 1). A type culture has been sent to the National Collection of Type Cultures, Lister Institute.

When a culture of *D. giganteum* is compared with *D. mucoroides* (Pl. 1, figs. 1, 2) it is always found that, comparatively, very few sorocarps are produced by *D. giganteum*, most of the myxamoebae being sacrificed in the production of long creeping stalks. No migration of the pseudoplasmodium could be seen in *D. giganteum* such as was described by Raper (1935) for *D. discoideum*. Sometimes (as shown in Text-fig. 1) a small secondary sorocarp with generally a rounded sorus is seen attached to the sorus, although this type of arrangement is uncommon and cannot be produced at will.

D. giganteum has been isolated only twice, on both occasions from decomposing compost heaps. The production of very long creeping stalks may be of importance in the dispersal of the spores in such substrates. No marked differences from *D. mucoroides* in such characters as size of myxamoebae and spores could be observed.

On several occasions a species whose sorocarps are shorter than *D. mucoroides* has been isolated from soils (Text-fig. 1). It is not proposed to give it a name unless similar observations have been made by other workers under controlled cultural conditions and with a range of suitable bacterial associates. No detailed study of the life cycle of this species has yet been made. It resembles *D. mucoroides* in the general outlines of its life cycle and in the size of sori but it differs in the maximum dimensions of the sorocarps when grown under controlled conditions and with suitable bacterial associates on non-nutrient agar. Text-fig. 1 gives a diagrammatic representation of the species of the genus *Dictyostelium* which can easily be identified.

Occurrence of Dictyostelium *spp. in the soils of Great Britain*

To study the occurrence of *Dictyostelium* spp. in soils, samples were taken from the top 4 in. The method of isolation and culture was that already described. It seems that only two species, *D. mucoroides* and *Dictyostelium* sp., are commonly present in the soils of England, Wales and Scotland, though only a few samples have been so far examined from Scotland. Other easily identifiable species such as *D. discoideum*, *D. minutum*, *D. purpureum*, *Polysphondylium violaceum* and *P. pallidum* have not been isolated from these soils. It seems strange that only two species of *Dictyostelium* have been found so far in British soils, and the same is true of the distribution of lytic myxobacteria (Singh, 1947).

Sixty-seven samples of arable and grassland soils from Hertfordshire, Berkshire, Bedfordshire, Wiltshire, Kent, Cornwall, Glamorganshire, Breconshire, Pembrokeshire and Aberdeen were examined. *Dictyostelium* spp. were found to be present in thirty-three of thirty-eight arable soils, and absent in five. Of twenty-nine grassland soils examined only three contained *Dictyostelium* spp. Two soil samples at a distance of 30 yd. from each other were collected on Harpenden Common. One soil had been under arable cultivation for 2 years and the other was permanent pasture; the cultivated soil gave plenty of fruiting bodies of *D. mucoroides* but none could be obtained from the soil under permanent grass. Miss F. J. Moore of the Plant Pathology Department, Rothamsted, found *Dictyostelium* fruiting bodies developing on potatoes infected with fungus when they were left in the laboratory for a few days.

No correlation between the pH of the soil and the distribution of *Dictyostelium* spp. was found in soils ranging from pH 4·3 to 7·8.

In addition to the soil samples mentioned above, the soils of all the classical plots of Barnfield and Broadbalk at Rothamsted were examined. Some of these soils have been treated with farmyard manure, some with artificial fertilizers only and some have been untreated. *Dictyostelium* spp. were found in all the plots. The presence of *Dictyostelium* species in these classical plots of Rothamsted where no fertilizer has been used, or only artificials have been added for 100 years or more, disproves the earlier belief that species of *Dictyostelium* are dung organisms. Their occurrence in dung merely indicates the presence of the bacterial food necessary for their development.

Counts of Dictyostelium *spp. in soil*

No satisfactory method of counting either the myxamoebae or the spores in soil has been developed. It is impossible to count myxamoebae directly because they are indistinguishable from the true small amoebae of the soil. The presence or absence of myxamoebae or of the spores at various dilutions can only be ascertained by the presence or absence of pseudoplasmodia and fruiting bodies. With the dilution method of counting holozoic Protozoa (Singh, 1946*a*,*b*) using *Aerobacter* strain 1912 as food on non-nutrient agar, the fruiting bodies of *Dictyostelium* spp. could be seen in cultures at several dilutions but their formation was so erratic that it was impossible to count the numbers either of myxamoebae or of spores.

DISCUSSION

The importance of Acrasieae in soil economy depends, first, on whether the myxamoebae can lead a trophic existence in soil and diminish the bacterial population, and, secondly, on their numbers. Some work has already shown that myxamoebae of two species of *Dictyostelium* migrate through sterilized soil and decrease the bacterial population rapidly. The formation of fruiting bodies takes place only at higher moisture contents (cf. Singh, 1946*c*). As both myxamoebae and the true amoebae which commonly occur in soil feed selectively on bacteria they may be important agents in bringing about qualitative changes in the bacterial flora of the soil.

This work was made possible by a grant from the Agricultural Research Council, to whom the author's thanks are due.

My best thanks are due to Dr H. G. Thornton, F.R.S., and Miss L. M. Crump for their interest in this work. Most of the strains of soil bacteria were kindly given to me by Miss L. M. Crump from her personal collection.

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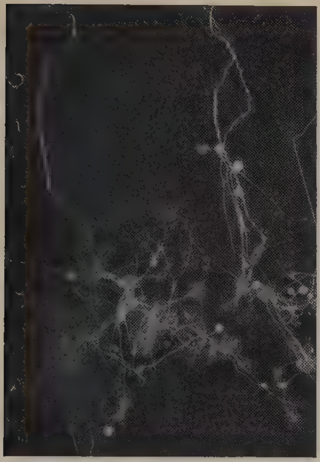

Fig. 1

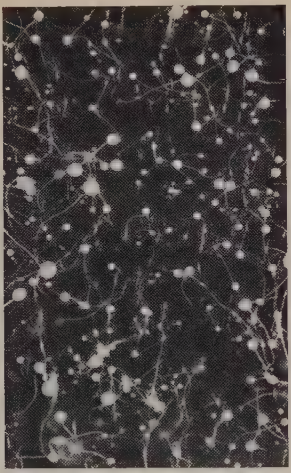

Fig. 2

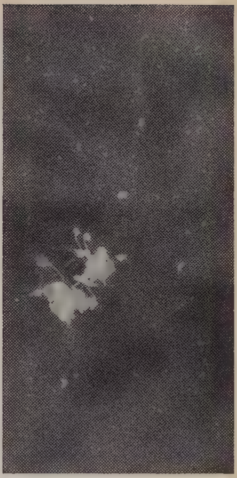

Fig. 3

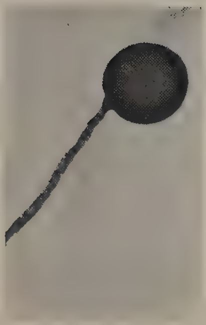

Fig. 4

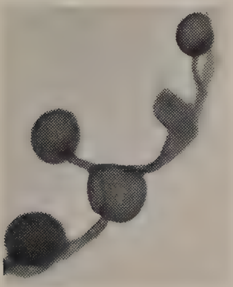

Fig. 5

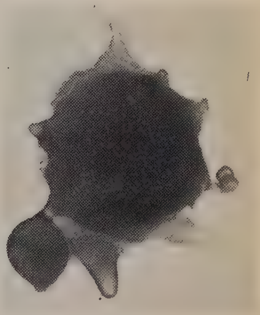

Fig. 6

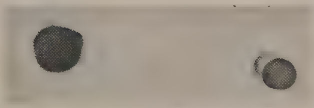

Fig. 7

B. N. Singh—Studies on soil acrasieae. Plate 1

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EXPLANATION OF PLATE

Fig. 1. *Dictyostelium giganteum* sp.nov., grown on non-nutrient agar and with *Aerobacter* sp. (strain 1912) as food supply. Only a part of the sorophores can be seen in the photograph.

Fig. 2. *Dictyostelium mucoroides* grown under similar conditions and with the same bacterial food supply as *D. giganteum*.

Fig. 3. Abnormal growth of *D. mucoroides* grown on non-nutrient agar with a yellow strain (6699) of soil bacteria as food supply. There is incomplete clearing of the bacterial patch and the sorophores are deformed and clumped in the centre. The fruiting bodies produced in other places have no stalks.

Figs. 1–3 are of the same magnification. (×6.)

Fig. 4. Showing a normal fruiting body of *D. mucoroides* grown on non-nutrient agar and with *Aerobacter* sp. as food supply.

Fig. 5. Showing abnormal fruiting bodies of *D. mucoroides* grown on non-nutrient agar with a red bacterium (strain 0312) as food supply.

Figs. 6, 7. Showing abnormal fruiting bodies of *D. mucoroides* grown on non-nutrient agar with a yellow bacterium (strain 6699) as food supply.

Figs. 4–7 are of the same magnification. (×50.)

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The Life Cycle of Sporing *Actinomyces* as revealed by a Study of their Structure and Septation

By EMMY KLIENEBERGER-NOBEL

From the Bacteriological Department, Lister Institute, London

SUMMARY: A study of the life cycle of four sporing Actinomycetes by Robinow's methods for the demonstration of Feulgen-positive chromatinic structures and of bacterial membranes, reveals two phases of growth in these organisms differing markedly in their morphological structure. These are: the primary or substratum mycelium which, by a special process, produces characteristic cells called 'initial cells', and the secondary or aerial mycelium which arises from the initial cells. The nuclear structures of the secondary mycelium undergo a division by which small rod- or dumbbell-shaped structures arise. Two of these appear to fuse, forming the round spore nucleus which, together with cytoplasm and enclosing membrane, represents the spore. The spores in their turn reproduce the primary mycelium.

Recent improvements in the staining of nuclear material and of the membranes and septa of bacteria warrant a re-examination of the changes which these elements undergo during the life cycle of related species or groups. It was with a view to finding new criteria for the purposes of classification that a study of the nuclear structures and septation in *Actinomyces* was undertaken. So far only a few of these spore-bearing organisms have been examined, but some constant morphological features of their developmental cycle have emerged and are here placed on record.

METHODS OF FIXING AND STAINING

The nuclear staining technique employed was introduced as a general method for the demonstration of chromatinic material by Robinow (1942, 1944), and applied to certain spore-bearing bacteria by Klieneberger-Nobel (1945). It consists of the treatment of cells fixed in osmic acid with N-HCl, followed by staining with Giemsa solution. The optimal temperature and time for the acid treatment, as well as the time for staining, varies with different organisms and their stage of growth and are best found by trial. For the *Actinomyces* the acid was usually applied for 6–20 min. at 55°, the Giemsa stain, diluted 1 in 30, for about 5–30 min. The specimens were mounted, either in the weak staining solution or in Canada balsam after dehydration with acetone and xylene. In the balsam mounts, the nuclear material stands out with great brilliancy; in watery solutions the cells as a whole are better preserved and cell boundaries as well as cytoplasm are more distinct. In some cases the chromatinic structures showed up clearly when stained for a short time in Giemsa stain only.

The cell boundaries, though occasionally visible in the HCl-Giemsa preparation, are better demonstrated by Robinow's method for the staining of membranes (1944, p. 420). Osmic-acid-fixed preparations were immersed in 5% watery solution of tannic acid for 30 min., well rinsed in several changes of water, stained for 2–4 min. in crystal violet 1 : 10,000, and mounted in the stain or in water. Membranes show up well if treated by this method, though, owing to the precipitation of the tannic acid on the membranous parts, they appear larger than they really are.

ORGANISMS STUDIED

A. gardneri, N.C.T.C. 6531: spores formed in long chains and whole hyphae transformed into spores.

A. albosporeus, N.C.T.C. 1578: spores mainly formed in the often spirally wound terminal parts of hyphae.

A. chromogenes, N.C.T.C. 1569: only side branches of hyphae form spores.

A. madurae, N.C.T.C. 1070: spores formed by short filaments only.

PREPARATION OF SPECIMENS

Czapek's medium was mainly used. The addition of a hay decoction (probably rich in amino-acids) greatly enhanced spore formation. In liquid medium coverslips were stood vertically in wide test-tubes, the lower half immersed in the liquid. Some cultures formed flat colonies on the glass surface and thus, on the borderline between the liquid surface and the air, stages of the whole growth cycle up to the formation of the spores were visible on the coverslip. Better preparations were obtained from solid media. An agar plate was inoculated with a spore suspension—dense if young and dilute if older developmental stages were required. Squares of the agar considerably smaller than the coverslip were placed, inoculated side downwards, on to the slip, and a drop of liquid Czapek medium was allowed to run round the edge of the square. The coverslips thus prepared were placed on filter paper in Petri dishes, which were incubated in a moist chamber. In the course of some days an even growth developed in the moist zone round the agar square. The growth, which adhered firmly to the glass surface after the removal of the agar, was fixed in the wet state and after drying was stained as described. It must be emphasized that the success of these preparations depends on the flatness of the growth on the glass surface, for the brilliancy of the staining as well as the definition of the structures are greatly enhanced by the thinness of the film. The low power photograph in Pl. 1, fig. 6, shows the excellent definition obtained over large fields in such preparations.

The optical system used was the same as in the previous work (1945). All photographs were taken by means of the Zeiss photographic eyepiece 'Phoku 9 : 12'.

THE LIFE CYCLE OF SPORE-BEARING ACTINOMYCETES

It has been known for a long time that two different phases of growth occur in all spore-forming Actinomycetes, which were designated by Ørskov (1923) as substratum mycelium and aerial mycelium. The substratum mycelium, henceforth referred to as primary mycelium, invariably develops from the spores, but never produces spores itself without first giving rise to an aerial or secondary mycelium, which is either wholly or partly transformed into spores.

It was found that the primary and secondary mycelia, which differ both in their growth requirements and habit of their filaments, are also conspicuously different in their structural make up.

It was observed that the secondary mycelium was invariably initiated in one particular element, which will be referred to as 'initial cell'. When the secondary hyphae have formed, spore formation, again involving particular structural changes, takes place. The life cycle of the spore-bearing Actinomycetes so far studied is therefore described under the following headings: A. The primary mycelium; B. The origin of the 'initial cells'; C. The secondary mycelium; D. The formation of the spores.

A. *The primary mycelium*

The development as far as it is similar in the four organisms examined will be described generally. Features characteristic of one or more organisms will be noted separately.

The mature spore (see below) contains one spherical, relatively large chromatinic body which is surrounded by cytoplasm enclosed in a spore case. One to three, and rarely four, tubules sprout in succession from the spores. The first tubules are always very slender, and irregularly undulating and twisted, a feature often found in fungal hyphae, but not in bacteria. In *A. gardneri*, *A. albosporeus* and *A. chromogenes* the germinating spore is slightly swollen and often elongated and its 'nucleus' has divided into two chromatinic structures (Text-figs. 1, 2 and Pl. 1, figs. 1, 2). In *A. madurae* the germinating spore is a round swollen body. When the young tubules have reached a certain size they contain small chromatinic granules presumably derived from the two structures found in the germinating spore. These, however, are not wholly used up in the process of germination but are still conspicuous in the spore when the primary mycelium is developed (Text-figs. 3, 4).

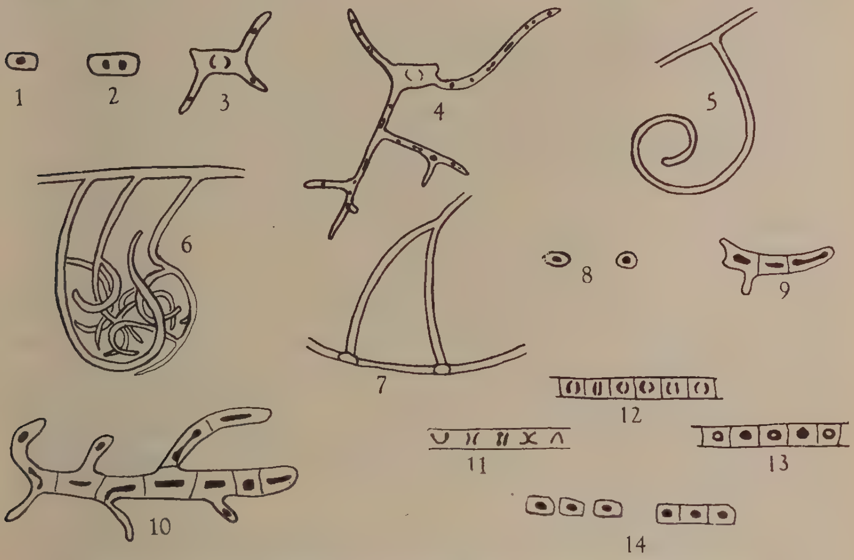
As the tubules grow the small nuclear bodies multiply so that the young mycelium is filled with granular or rod-shaped chromatinic bodies (Text-fig. 4 and Pl. 1, fig. 4). Owing to the smallness of these structures no indication could be obtained as to whether they divide by simple fission or by splitting lengthwise in the manner of chromosomes.

In the stages represented by Pl. 1, fig. 3, there are not yet any indications of transverse septa. Nevertheless, septa are formed early in primary mycelia, but they can be demonstrated only by the tannic acid-crystal violet method. Transverse septa seem to originate from small condensations in the outer cell membranes both in the young primary mycelia (Pl. 1, fig. 5) and the secondary mycelia (Pl. 3, fig. 25). Ørskov described this development in secondary mycelia but did not observe it in the primary perhaps owing to the less perfect staining by the less adequate methods then available. In Pl. 1, fig. 5, the spore that gave rise to one of the young primary mycelia is to be seen; some of the transverse septa are fully developed, others are only rudimentary. In older hyphae septa are more widely spaced.

Though primary mycelia are septate they never break up into single cells and in this respect differ sharply from the secondary mycelia.

B. *The origin of the 'initial cells'*

When the primary mycelium has reached a certain stage of development, agglomerations of hyphae appear in the form of skeins, network and scrolls. In *A. gardneri* they are compact, nest-like, in others they consist mainly of rolled up end- and side-branches. When *A. gardneri* is grown on the hay decoction medium which encourages spore formation, the filamentous nests appear on the second day of growth and are conspicuous under low magnification (Pl. 1, figs. 9, 10, and Text-figs. 5, 6). In these areas new cellular elements arise at the points where two filaments are in close contact (Text-fig. 7). In the compact nests of *A. gardneri* the first changes that can be detected are usually darkly staining nuclear granules standing out from the tangled filaments. These granules, which represent the rudiments of the new cells, are surrounded



Text-figs. 1–14, outlining the life cycle of spore-bearing Actinomycetes.

Text-fig. 1. Resting spore.
Text-fig. 2. Germinating spore. (Type *A. gardneri* or *albosporeus*.)
Text-fig. 3. Three germination tubules have been formed.
Text-fig. 4. Young primary mycelium, spore still visible. (Septa not drawn.)
Text-figs. 5, 6. Filaments in 'nest'-formation. (Septa and nuclear structures not drawn.)
Text-fig. 7. Arising of initial cells. (Septa and nuclear structures not drawn.)
Text-fig. 8. Initial cells.
Text-figs. 9, 10. Young secondary mycelia.
Text-fig. 11. Nuclear cylinders of secondary hyphae have split up into 'chromosomes'.
Text-fig. 12. Pairs of 'chromosomes' are going to form the spore nuclei.
Text-fig. 13. Newly formed spores still connected with each other.
Text-fig. 14. Mature spores.

by delicately staining cytoplasm and later on develop a membranous outline. Occasionally small 'nests' are found with only a few new cells in the process of formation. In Pl. 2, figs. 14 and 15, two and four respectively of these new cells are to be seen in the centre of converging filaments. Pl. 2, fig. 16, shows a cluster of such new cells in some of which the chromatinic body and the surrounding cytoplasm are clearly visible (see also Text-fig. 8). When these cells are fully developed, the filaments from which they arose stain feebly and seem to fade away. The newly formed, brightly staining 'initial cells' elongate and grow into the secondary mycelium.

The second type of initiation can be seen, starting during the first day of incubation, in a vigorously growing culture of *A. madurae*. The filaments either roll up into a scroll and entangle, or very short side branches bend round until their tips make contact with the main branch from which they sprang, thus producing small loops (Text-figs. 5, 6 and Pl. 1, figs. 7, 8). Though the rolling up is the rule in the strain of *A. madurae* studied, network and skein-formation occur as well. Thus a new element may form where two separate cells touch one another as in Pl. 2, fig. 11, where two nodes representing the rudiments of two 'initial cells' have arisen. When these cells elongate and are still in rigid connexion with their parent filaments they are forced to grow into a loop (Pl. 2, fig. 12). If one of the interconnexions gives way (for at that stage the primary mycelium seems to be fragile), the young secondary filament unbends, forming first a hook and then a straight, wide, darkly staining filament (Pl. 2, fig. 13). Initial cells often become wholly separated from the primary mycelium, growing independently to form secondary mycelia on top of the decaying primary filaments.

The condensation of filaments described above is a constant feature in the four species of *Actinomyces* so far examined. It is seen first at the centre of the colony and later at its periphery. Wherever it arises initial cells develop; these in turn develop into secondary mycelia.

C. *The secondary mycelium*

When the initial cell elongates, its deeply staining chromatinic body develops into an oval or cylindrical structure. This, like the whole cell, seems to divide by a process of fission, forming rows of oval or cylindrical cells. Pl. 2, fig. 18, shows a nest in the centre and rows of brightly stained secondary cells lying on top of the fading primary mycelium in *A. chromogenes*. Here the secondary mycelium is the only conspicuous type of growth. The fully grown hyphae consist of long cells with characteristic, deeply staining chromatinic cylinders, closely resembling the so-called 'fusion cell' of spore-bearing bacilli (Klieneberger-Nobel, 1945). Cells of secondary hyphae of *A. albosporeus* are depicted in Pl. 2, figs. 17, 20, and of *A. gardneri* in Pl. 2, fig. 21.

These mycelia are characterized not only by their chromatinic cylinders but also by their easily stainable transverse septa, which are usually visible in the HCl-Giemsa preparation (Pl. 2, figs. 17, 20) and very conspicuous in preparations stained for cell membranes. Long compartments of a fully grown hypha separated by well-defined transverse septa can be seen in Pl. 2, fig. 19. The secondary mycelia are often composed of fairly straight, diverging filaments with fewer side branches than primary mycelia. The side branches, which are produced by sprouting, are slender at first and their nuclear structures often not yet clearly developed, but when fully grown they always resemble the main branch. In Text-figs. 9 and 10, young secondary mycelia are drawn as they appear when stained for nuclei. In Pl. 2, fig. 22, two young secondary mycelia are shown as they appear when stained for cell walls. It should be mentioned that in such preparations the tips of filaments and sprouts are often darkly stained, suggesting a condensation of the membranous substances. Since the secondary hyphae are distinctly septate it is not surprising that they break up readily, and in some organisms, for example in *A. gardneri*, they usually break up into single cells (Pl. 2, fig. 21) which have the same structure and growth potentialities as the cells of unbroken filaments.

D. *The formation of the spores*

The chromatin cylinders of the secondary hyphae change in a characteristic way before the spores are formed. At first they show irregular intersections, and as the divisions develop a more or less regular pattern appears (Pl. 2, fig. 23 and Pl. 3, figs. 24, 28). As outlined diagrammatically in Text-fig. 11, short chromatinic filaments or dumbbells appear lying across the longer axis of the cells. They often occur in pairs, either lying parallel to one another, or in a V- or X-configuration. In their shape, situation in the cell and arrangement in pairs, these structures strongly resemble the so-called dumbbell bodies or 'chromosomes' of bacteria (Neumann, 1941; Robinow, 1942, 1944; Klieneberger-Nobel, 1945). They are not always clearly demonstrable; sometimes only open loops can be detected; but repeated examinations of similar material provides convincing evidence that division of the chromatinic material occurs regularly whenever spores are being formed. The short filaments, dumbbell bodies or 'chromosomes' set free by this process of division seem to be the elements which later on make up the spore nuclei. It is difficult to decide whether one or two of these elements go to form the chromatin body of each spore, but, since later stages often show two crescent-shaped chromatinic bodies almost forming a ring, it seems justifiable to conclude that two elements go to form the spore nucleus. In the young spore the chromatinic substance usually appears to be disk-shaped with a ring-like condensation of nuclear material at the edge (Pl. 3, fig. 26); later probably by condensation it takes the shape of a sphere (Pl. 3, figs. 27, 29, 30). The changes leading to the formation of the spore are outlined in Text-figs. 12, 13 and 14.

When the round spore nuclei are being formed in the secondary hyphae, new septa start to develop, subdividing the large compartments into smaller ones as indicated in Pl. 3, fig. 25, and completed in Pl. 3, fig. 33. That each small compartment corresponds to one single spore cell can be seen when fig. 31 is compared to fig. 33 in Pl. 3. Each shows a different sporing side-branch of *A. chromogenes* prepared from the same series of coverslip cultures; in fig. 33 the septa, in fig. 31 the nuclei have been stained. It will be seen that in approximately the same length of filament the same number of compartments and nuclei is found. Faintly stained septa of spore compartments can also be detected in Pl. 3, figs. 29, 30, illustrating spores stained for nuclei, while in Pl. 3, fig. 32, two sporing hyphae stained for cell walls are shown; here the septa of the spore cells are very distinct. In the right-hand hypha some cells are in the process of separation and show two membranous borders at the point where they break. Thus each single spore, consisting of a round chromatinic body and surrounding cytoplasm, appears to be enclosed in a spore case.

DISCUSSION

Most investigators of Actinomycetes have used either stained smears or living material for their studies. As Ørkov pointed out in 1923, smears are very unsatisfactory for elucidating the complex growth of the ray fungi, on account of tearing and disintegrating the whole culture. On the other hand his improved method of studying the growth on the agar surface—rightly followed by many later workers (see Erikson, 1935)—though not dislocating the culture, does not allow the demonstration of cellular structures, so important for the recognition of a particular stage of growth. The methods used here avoid dislocation and at the same time permit the differentiation of chromatinic bodies, cytoplasm and membranes. Regularly arranged chromatinic structures have hitherto been observed by Badian (1936) and von Plotho (1940). Schaede (1939) was unable to demonstrate any nuclear material in Actinomycetes. Badian was concerned only with the chromatinic material and did not study septation. His interpretation of the changes in the chromatinic matter during development is somewhat speculative and his evidence is not very convincing. Von Plotho, though not realizing the whole complexity of the cycle, described a Feulgen-positive substance in Actinomycetes which concentrated and divided during spore formation. She also observed septation in sporing hyphae. Her description and photomicrographs are in good agreement with those presented above.

Apart from these papers there is little in the literature about a regular cell morphology in Actinomycetes. Two conceptions, which appear to have been generally accepted, may be mentioned. Lieske's and Ørskov's view, that the primary mycelium of Ørskov's group I, comprising the spore-forming organisms, is unicellular, has not been confirmed. Though the primary mycelium does not break up and the septa can be demonstrated neither in the living state, nor when stained without the preceding application of a mordant, transverse septa are undoubtedly present. Secondly, the description of spore formation as found in Lieske's and Ørskov's monographs, that 'Spores are formed from the aerial hyphae without any previous segmentation of the cytoplasm' (Topley & Wilson, 1946) can no longer stand; for during spore formation the hyphae are separated by transverse septa into small cells, each of which will develop into a spore.

The main features of the developmental cycle of the Actinomycetes studied are outlined in the Text-figs. 1–14, and appear to be as follows: The spore consists of a round chromatinic body, cytoplasm and a spore case; it germinates by sprouting. Its nuclear body divides into two structures, and partition products of these enter into the germination tubules. The primary mycelium develops further by stretching and sprouting accompanied by division of its small nuclear bodies which are found in all side branches. Eventually the whole primary mycelium is composed of compartments of different length divided from each other by delicate transverse septa and containing probably a variable number of small rod-like or spherical chromatinic bodies. The hyphae of the much-branched primary mycelium are usually more twisted and undulating than those of the secondary mycelium.

As soon as it is well developed the young primary mycelium produces agglomerations of filaments in many areas, first in the centre of the colony and later at the periphery. Where two parts of one filament or two different filaments touch each other new elements arise, which consist of darkly staining nuclear bodies surrounded by cytoplasm and later on enclosed by a cell wall (cf. Lieske's (1921) 'Vierhyphensporen'). For these the name 'initial cells' is proposed since they initiate the secondary mycelium. It is probable that at the points where two filaments make contact with each other the content of two cells, belonging originally to two different hyphae or parts of hyphae, fuse together, so that the initial cell may be regarded as a fusion cell and, in particular, its chromatinic body as a fusion nucleus. Though it was not possible to follow up the process of fusion itself, both the 'nest' formation and the structure of the secondary mycelium favour such an assumption. The elements of which the secondary mycelium is built up closely resemble the so-called fusion cells previously described for certain Clostridia and *Bacillus mycoides*. They differ however in their origin. In the bacteria the fusion of the chromatinic material takes place in one and the same cellular compartment while in *Actinomyces* it is supposed that two nuclei or contents of different cells melt together to form the initial cell.

The initial cells grow into the secondary mycelium by a process of sprouting and subdivision of cells. The secondary hyphae are composed of fairly long, cylindrical cells each of which contains a big deeply staining, cylindrical mass of nuclear material. The transverse septa, subdividing the hyphae, are very well defined and easily demonstrable. The hyphae often break up into single cells, particularly in certain species and on certain media. These single cells have exactly the same structure as those of the hyphae and they are able either to grow out again into a new secondary mycelium or to form spores. The conception of the primary mycelia as a haploid phase and the secondary a diploid phase is a promising speculation but in order to prove this an analysis of 'chromosome' content complemented by observations proving the actual fusion of chromatin would be required. Such evidence will be difficult to obtain, since all the elements concerned are of exceedingly small dimensions.

Spore formation begins by division of the nuclear cylinders in the cells of the secondary mycelium. The products of this nuclear division are of definite shape and their arrangement seems to follow a regular pattern. Whereas the nuclear cylinders occupy a longitudinal position in the cells, the small rod-like or dumbbell structures derived from them lie across the cells, singly, but more often in pairs, either parallel to one another, or in V- or X-like configurations. Just as the cell of the secondary mycelium resembles the fusion cell of certain spore-bearing bacteria, the small rod-like structures resemble the bacterial 'chromosomes' seen in both the dividing cell and spore formation. It is therefore justifiable to conclude that, unlike the primary mycelium the secondary mycelium has in common with spore-bearing bacteria certain features such as the chromatinic cylinder in the vegetative cell and the chromosome-like rods with their regular arrangement in the spore-forming stage. On the other hand the secondary mycelia of *Actinomyces* differ from the spore-forming bacteria in their multiplication by sprouting and in the actual formation of the spores. In the spore mother cell of spore-forming bacteria one 'chromosome' develops into the spore nucleus, and the three remaining ones are extruded. In the spore-forming *Actinomyces* the 'chromosomes' appear to be used up in the process of spore formation, one pair being necessary—if the difficult observations have been rightly interpreted—for the formation of a disk-like or spherical nucleus. In bacteria the big single spore chromosome is at first an open loop and later a disk-like structure before it takes its ultimate position and shape as described by Robinow (1942). Formation of new septa takes place in both groups of organisms; in bacteria, to separate the spore cell from the remainder of the mother cell, in *Actinomyces* to separate each spore cell from its neighbour in the filament.

The newer methods for the demonstration of cellular structures in bacteria have revealed new features in Actinomycetes, contributing to a better understanding of their life cycle. It is probable that application of the methods to many more species and groups of organisms will shed new light on their position in a natural system of classification of microbes.

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EXPLANATION OF PLATES

Plate 1

Figs. 1, 2. *A. gardneri*; spores germinating after 8 hr. at room temperature; HCl-Giemsa. × 2000.

Fig. 3. *A. gardneri*; spores germinating; tannic acid-crystal violet. × 2000.

Fig. 4. *A. gardneri*; young primary mycelium; HCl-Giemsa. × 2000.

Fig. 5. *A. madurae*; primary mycelia, stained for membranes; note original spore in lower left corner; tannic acid-crystal violet. × 2000.

Fig. 6. *A: albosporeus*; secondary hyphae with nuclear cylindrical structures just detectable at low magnification; HCl-Giemsa. × 500.

Figs. 7, 8. *A. madurae*; primary mycelia forming agglomerations of filaments; Giemsa. × 2000.

Figs. 9, 10. *A. gardneri*; primary mycelia with 'nests' of tangled filaments; Giemsa. × 500.

Plate 2

Fig. 11. *A. madurae*; two initial cells developing on primary mycelium; Giemsa. × 2000.

Fig. 12. *A. madurae*; two initial cells growing into loop-shaped secondary hyphae; Giemsa. × 2000.

Fig. 13. *A. madurae*; young secondary hyphae unbend and elongate; Giemsa. × 2000.

Fig. 14. *A. gardneri*; rudiments of two initial cells; HCl-Giemsa. × 2000.

Fig. 15. *A. gardneri*; rudiments of four initial cells in the centre of converging primary hyphae; HCl-Giemsa. × 2000.

Fig. 16. *A. gardneri*; young initial cells in nest-like configuration; HCl-Giemsa. × 2000.

Fig. 17. *A. albosporeus*; secondary hypha; HCl-Giemsa. × 2000.

Fig. 18. *A. chromogenes*; initial cells lying on top of primary mycelium and growing into secondary hyphae; HCl-Giemsa. × 2000.

Fig. 19. *A. albosporeus*; secondary hypha showing septation; tannic acid-crystal violet. × 2000.

Fig. 20. *A. albosporeus*; secondary hypha showing nuclear cylinders and transverse septa; HCl-Giemsa. × 2000.

Fig. 21. *A. gardneri*; secondary hypha broken up into single cells; HCl-Giemsa. × 2000.

Fig. 22. *A. gardneri*; two young sprouting secondary mycelia; tannic acid-crystal violet. × 2000.

Fig. 23. *A. albosporeus*; the nuclear cylinders have divided up into small filamentous structures which lie across the cells ('chromosome stage'); HCl-Giemsa. × 2000.

Plate 3

Fig. 24. *A. albosporeus*; 'chromosome stage' as in 23; but not yet quite as well separated; HCl-Giemsa. × 2000.

Fig. 25. *A. albosporeus*; secondary mycelium showing septation; note the rudiments of new septa which indicate that spores are being formed; tannic acid-crystal violet. × 2000.

Fig. 26. *A. albosporeus*; two chromatinic filaments or 'chromosomes' seem to form one ring-shaped nucleus each, note also transverse septa; HCl-Giemsa. × 2000.

Fig. 27. *A. gardneri*; secondary mycelium in which the round spore nuclei have developed; HCl-Giemsa. × 2000.

Fig. 28. *A. albosporeus*; 'chromosome stage', note the two crescent-shaped filaments which seem to be drawn apart; HCl-Giemsa. × 2000.

Fig. 29. *A. gardneri*; the whole secondary mycelium has been transformed into spores; HCl-Giemsa. × 2000.

Fig. 30. *A. albosporeus*; terminal branches of secondary mycelium have been transformed into spores; HCl-Giemsa. × 2000.

Fig. 31. *A. chromogenes*; a spore-bearing hypha showing nuclei; HCl-Giemsa. × 2000.

Fig. 32. *A. gardneri*; secondary mycelium; note two horizontal filaments which have divided up into short spore cells; tannic acid-crystal violet. × 1600.

Fig. 33. *A. chromogenes*; a spore-bearing hypha showing septation; tannic acid-crystal violet. × 2000.

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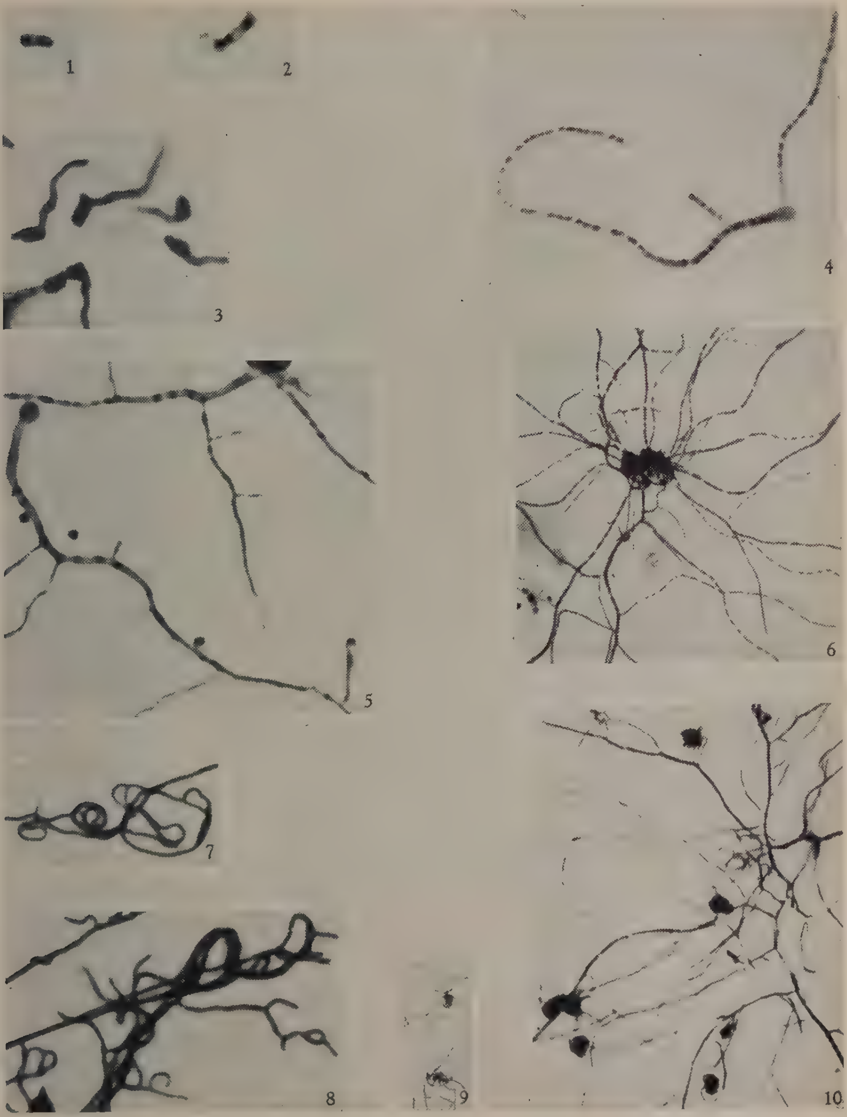
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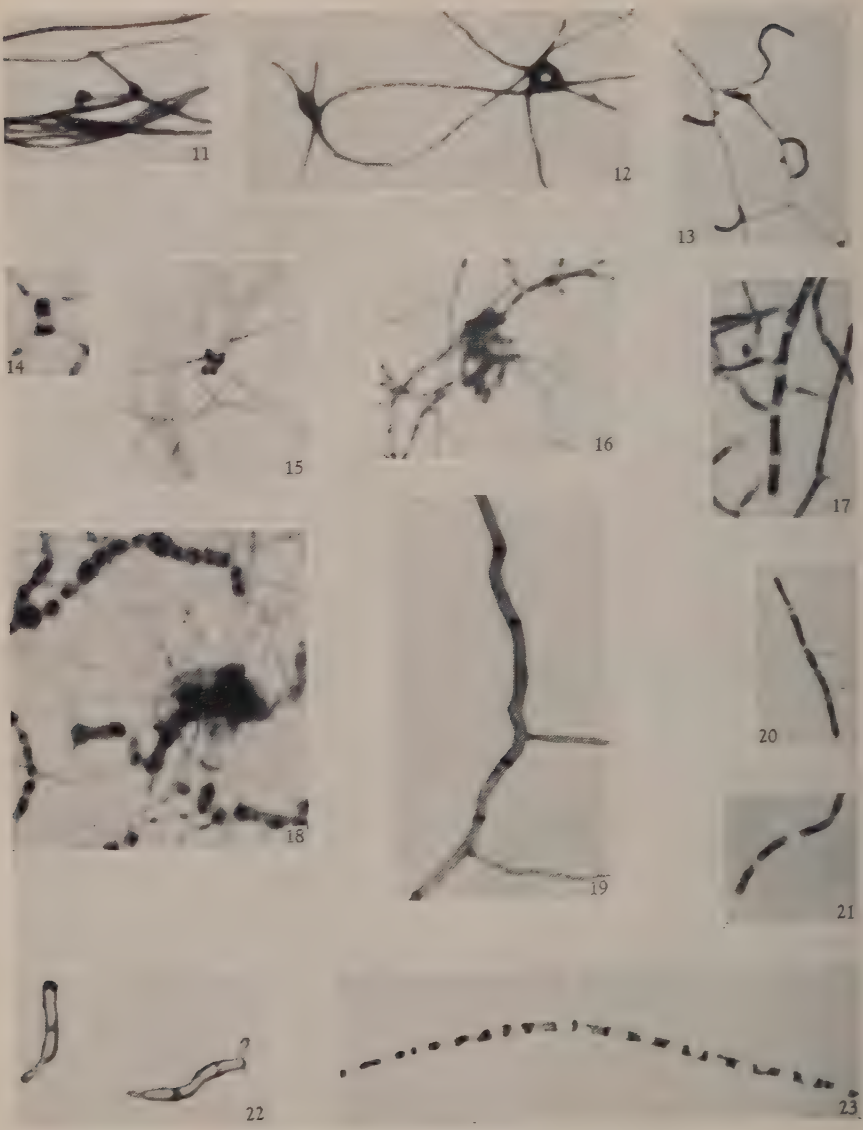
Figs. 1–10

E. Klieneberger-Nobel—Life cycle in *Actinomyces*. Plate 1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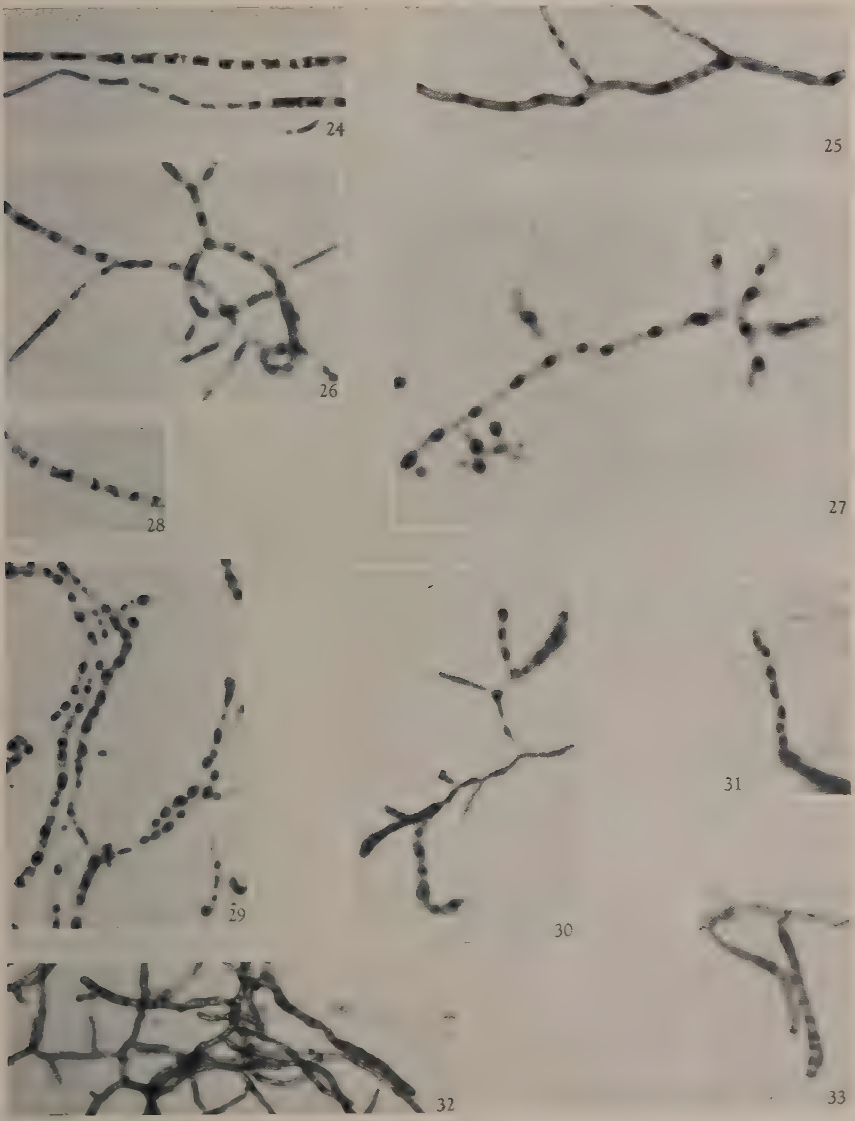
Figs. 11–23

E. Klieneberger-Nobel—Life cycle in *Actinomyces*. Plate 2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Figs. 24–33

E. Klieneberger-Nobel—Life cycle in *Actinomyces*. Plate 3

A Cytological Study of Myxococci

By EMMY KLIENEBERGER-NOBEL

From the Bacteriological Department, Lister Institute, London

SUMMARY: Myxococci are distinguished from other bacteria by the complete lack of a cell membrane as well as of transverse septa. Even the microcyst is enclosed by an outer layer which differs from a bacterial cell wall. In cytological character the young stages in the life cycle resemble other bacteria in so far as they contain two to four small nuclear structures or 'chromosomes' arranged transversely in the cell and dividing longitudinally. Older organisms about to form microcysts differ; they contain two fairly large nuclear structures which fuse to form a round chromatinic body. At the same time the cell shortens until a round organism containing one large, round, darkly staining nuclear body is formed. This 'fusion cell' can be compared to a zygote though it is not yet the resting cell of the species. The fusion cell becomes oval, its nucleus divides into two, and its outer layer becomes tough and dense. Thus the microcyst is formed. When it germinates its outer layer disappears, and the cell is transformed by elongation into the young vegetative organism.

A study has been made of the life cycle of myxococci by methods used in recent years for the fixing and staining of nuclear structures, cytoplasm and membranes in bacteria (Klieneberger-Nobel, 1945, 1947; Robinow, 1942, 1944, 1945). The organisms investigated were *Myxococcus fulvus*, *M. virescens* and *Chondrococcus exiguus*. They were kindly provided by Dr Singh of the Rothamsted Experimental Station.

METHODS

The methods employed are similar to those used in earlier work (Klieneberger-Nobel, 1945, 1947). The nuclear structures of young cells could be demonstrated by staining fixed preparations with Giemsa's solution. The more advanced stages, particularly the mature microcysts, needed the acid treatment before staining. The best growths for examination were obtained in the moist zone on the coverslip round the piece of agar. Here the cultures developed freely and were not disturbed when the agar was removed. A water agar enriched by a heat-killed suspension of *Bacterium coli* in saline served as the medium (Singh, 1946, 1947). Seitz-filtered glucose solution (Stanier, 1942*a*,*b*) and small amounts of dung extract were frequently added to promote growth. Softness of the agar was advantageous. The incubation temperatures varied between 25° and 34°. For the study of the germination of the microcysts it was necessary to incubate at temperatures slightly above 30° in order to ensure a quick and simultaneous development.

THE LIFE CYCLE

(*a*) *The germination of microcysts*

The mature, almost round microcyst occurs in the fruiting bodies of cultures 2–4 weeks old. It possesses a thick, dense outer layer which simulates a membrane and stains deeply. Two nuclear structures, which are usually in close contact, are demonstrable in mature microcysts after treatment with HCl (Text-fig. 15).

In old cultures varying numbers of disrupted cysts are frequently found. They appear to arise through an extrusion of the contents of the microcyst (Pl. 1, figs. 9, 10). The two nuclear structures or granules are distinguishable in the extruded material. When cultures on fresh media are examined at short intervals it is found that the disrupted cysts show no further development and that their number does not increase. They must therefore be regarded as dead. They might easily be mistaken for germinating cells if the mode of microcyst development was not known (see Beebe, 1941; Badian, 1930). Other forms in old cultures which show no further development are organisms which have not been able to complete their cycle by forming microcysts.

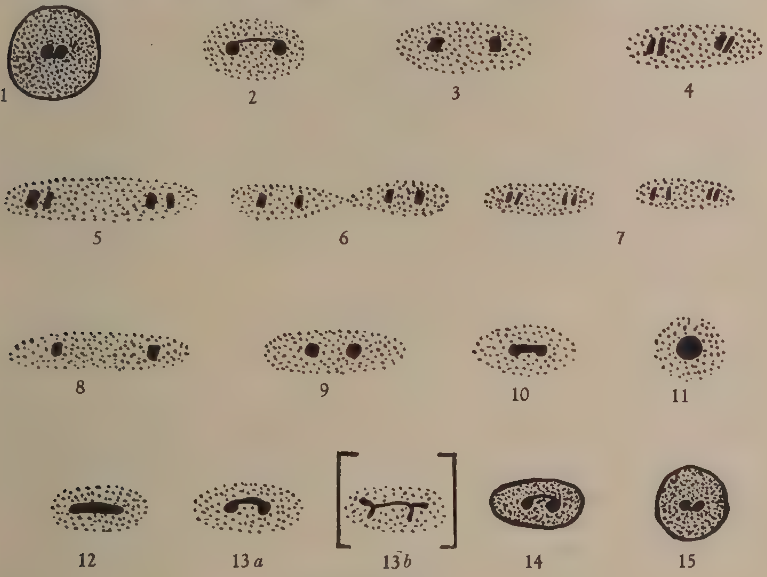
If mature microcysts are spread on agar and incubated so that they develop more or less simultaneously, germination can be observed clearly. At first the microcyst swells slightly and gradually acquires a transparency until its staining qualities are comparable to those of the young vegetative cell. At the same time its definite contours gradually become less distinct; its nuclear structures, which now appear bigger, more deeply stainable and conspicuous than in the resting cyst, move further apart (Text-figs. 1, 2). Up to this point the actual shape of the cell may not have changed noticeably, but from now onwards the cell begins to elongate (Text-fig. 2; Pl. 1, figs. 1–4), or occasionally to develop a finger-like protuberance (Pl. 1, figs. 3, 4). Thus the microcyst slowly transforms itself into the young bacillary form which possesses no membrane and contains two darkly staining nuclear structures (Text-fig. 3; Pl. 1, figs. 2, 7). On occasions a subdivision of the nuclear structures has already started in this very young stage and the bacilli show two pairs of nuclear structures (Text-fig. 4; Pl. 1, fig. 5, *a*). The mode of germination is not sudden; there is no bursting of a 'shell'; nothing is left behind when the young bacillus is formed. The whole process is a gradual one: the tough outer layer disappears, the cytoplasm becomes transparent, the nuclear structures swell and move apart and the cell elongates and takes on the bacillary shape. The first bacillary elements have been observed after 4–6 hr. of incubation and after 10–15 hr. the majority of microcysts have been transformed into rods.

(*b*) *The development of the young vegetative cells*

The young cells are slender organisms and do not possess a cell membrane proper or any transverse septa. These elements, which are so characteristic of the Eubacteriales, were never demonstrated in myxococci by means of Robinow's (1944) method for the staining of membranes, although tests were carried out repeatedly during the course of this study. The cells gradually divide into two by constriction (Text-fig. 6). When division has been completed the pointed ends of the resulting cells round off. Before a cell divides its two nuclear structures divide lengthwise into four bodies (Text-figs. 5, 7), which are often connected by a skein of chromatinic material until the constriction sets in. This deeply staining skein sometimes resembles a continuous nuclear band, but close scrutiny of delicately stained preparations reveals the fact that the young vegetative organisms contain two to four single chromatinic structures arranged more or less transversely to the axis of the cell (Pl. 1, figs. 5–8). In their arrangement and mode of division they closely resemble the so-called 'chromosomes' of other bacteria.

(c) *The formation of 'fusion cells'*

When a culture is well established and the young vegetative organisms have spread over the medium many cells start on a new development. The two nuclear structures of each cell increase in size (to about double) as if preparing for longitudinal division, yet they do not split but remain 'double-sized bodies' (Text-fig. 8). The cell now becomes shorter and the 'double-sized bodies' approach each other (Text-fig. 9; Pl. 1, figs. 11, 12; Pl. 2, fig. 13). Eventually they fuse and form a rod-like and later a round and conspicuous chromatinic structure (Text-figs. 10, 11; Pl. 2, figs. 14, 15). The cell shortens until finally a roundish cell with a round or ring-like nuclear body results (Text-figs. 10, 11). This cell, which I have called a 'fusion cell', might perhaps be regarded as the end stage of the cycle; yet it is not the resting stage, which arises from it by a further development. The fusion process occurs particularly in the centre of swarms of myxococcal cells that are collecting together in order to form the fruiting bodies.



Text-figs. 1–15.

(d) *The formation of the microcysts*

The nuclear body of the 'fusion cell' (Text-fig. 11) soon stretches and grows into a rod- or dumbbell-like structure (Text-figs. 12, 13*a*; Pl. 2, figs. 16, 17, 19). At the same time the whole cell enlarges and stretches to the oval form.

As development proceeds its nuclear structure divides into two round bodies which usually, but not always, remain connected by a string of chromatinic material (Text-fig. 14). At this stage the cell has acquired a greater affinity for dyes and at the same time formed a tough, dense outer layer. Thus the microcyst is formed (Pl. 2, figs. 16–18). With increasing age the microcyst becomes more round and smaller; its nuclear structures move more closely together and the whole cyst's affinity for dyes is now very pronounced (Text-fig. 15). In *Chondrococcus exiguus* the rearrangement of nuclear material that occurs during the maturation of the fusion cell suggests that a reduction of chromatin may take place. The cyst-forming cells often show a subdivision of their nuclear material into four structures instead of only two (Text-fig. 13*b*). It was, however, not possible to determine whether two of these four structures were finally eliminated, or if they joined up again to form the two chromatinic bodies found invariably in the mature microcyst, or if these cells were involution forms. The development of the fusion cell may be regarded as a step towards the development of an embryonic vegetative cell; for the nuclear apparatus of the young vegetative cell is already fully developed in the resting microcyst, just as the embryo is already present in the mature seeds of higher plants. In contrast to the vegetative forms the microcyst is resistant against drying and ageing. When it eventually germinates on a fresh medium the young vegetative form arises as described under (*a*).

DISCUSSION

There are only a few papers dealing with the cell morphology of myxococci. The first of these was published in 1910 by Vahle. He used osmic acid fixation and stained with methylene blue. He describes and illustrates the two nuclear structures of the vegetative cell and their subdivision into four in dividing cells. He has also seen two structures in the 'spores', or 'microcysts' as they are called now. As knowledge regarding nuclear structures in bacteria was in an uncertain state at that time it was not possible to link up Vahle's observations with findings in other organisms and his valuable contribution to the cytology of micro-organisms was lost. Four recent workers, the Krzemieniewski (1928), Badian (1930, 1933) and Beebe (1941), have taken up the study of the cytology and development of myxococci. Badian certainly observed and illustrated some of the obvious appearances of the myxococcal cell with its conspicuous nuclear structures, but he suggests a complicated scheme of transformations of the chromatinic material during the life cycle of the organism which have not been confirmed in the experiments here described. Beebe, as well as Badian, observed genuine nuclear structures in *Myxococcus xanthus*, but his scheme of the development appears obscure, and from his evidence (drawings and photographs) no definite and clear idea of the cycle can be formed. It seems certain that both Badian's and Beebe's descriptions and drawings concerning the germination of microcysts are at fault. The observations here reported show that the developmental cycle in myxococci follows a much simpler course than was conceived by these workers. The most thorough investigation in the morphology of myxococci so far made is that of the two

Krzemieniewski. Their observations are in excellent agreement with mine but were not worked out so as to suggest an outline of the life cycle of the organisms.

The present observations, together with others already reported, have brought to light a number of morphological characters in which endospore-forming bacteria, spore-forming actinomycetes and microcyst-forming myxococci exhibit both similarities and distinctions. It has been shown that the three groups of organisms have certain characteristics in common such as the chromatin distribution at some stage and the formation of a 'fusion cell', preceding the development of a resting stage.

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EXPLANATION OF PLATES

(Magnification ×3000)

Plate 1

Fig. 1. *Myxococcus fulvus*. Germinating microcysts.

Fig. 2. *Chondrococcus exiguus*. Germinating microcysts. The cysts are stretching to form the young bacillary forms.

Fig. 3. *Myxococcus fulvus*. Germinating microcysts. Note the chromatinic bodies that have moved apart and the forms that have stretched.

Fig. 4. *M. fulvus*. Germinating microcysts. Note the finger-like protuberances.

Figs. 5, 6. *M. fulvus*. Young bacillary stage showing two to four chromosomes. Note the organism at *a*.

Fig. 7. *Chondrococcus exiguus*. Young bacillary stage. The couple of chromosomes are dividing into four.

Fig. 8. *Myxococcus fulvus*. Young bacillary stage. The organisms are dividing rapidly and each round nuclear body corresponds to two chromosomes which can only occasionally be resolved.

Figs. 9, 10. *M. fulvus*. Burst microcysts.

Fig. 11. *M. virescens*. Older culture. The organisms are collecting in some places in order to form microcysts. Each organism contains two 'double-sized' nuclear structures.

Fig. 12. *M. fulvus*. Older culture. The organisms are slightly shortened and the two 'double-sized' chromosomes are approaching each other.

Plate 2

Fig. 13. *Myxococcus virescens*. Older culture. Some organisms are very short and their 'double-sized' chromosomes have almost fused.

Fig. 14. *M. virescens*. Older outgrowth showing 'fusion cells'; some are almost round.

Fig. 15. *M. fulvus*. Older growth showing various stages of chromatin fusion.

Fig. 16. *M. fulvus*. Microcyst formation. Notice some small round 'fusion cells' with one nuclear body and others that have grown into the oval form and show two nuclear bodies.

Fig. 17. *M. fulvus*. Microcyst formation, showing various stages in the development of the small round fusion cell with one chromatinic body to the oval mature microcyst with two chromatinic structures.

Fig. 18. *M. fulvus*. Part of fruiting body containing many microcysts.

Fig. 19. *M. fulvus*. Microcyst formation. Similar stages to those in Figs. 16 and 17. Note at *a* and *b* the stretched nuclear body which has not yet divided.

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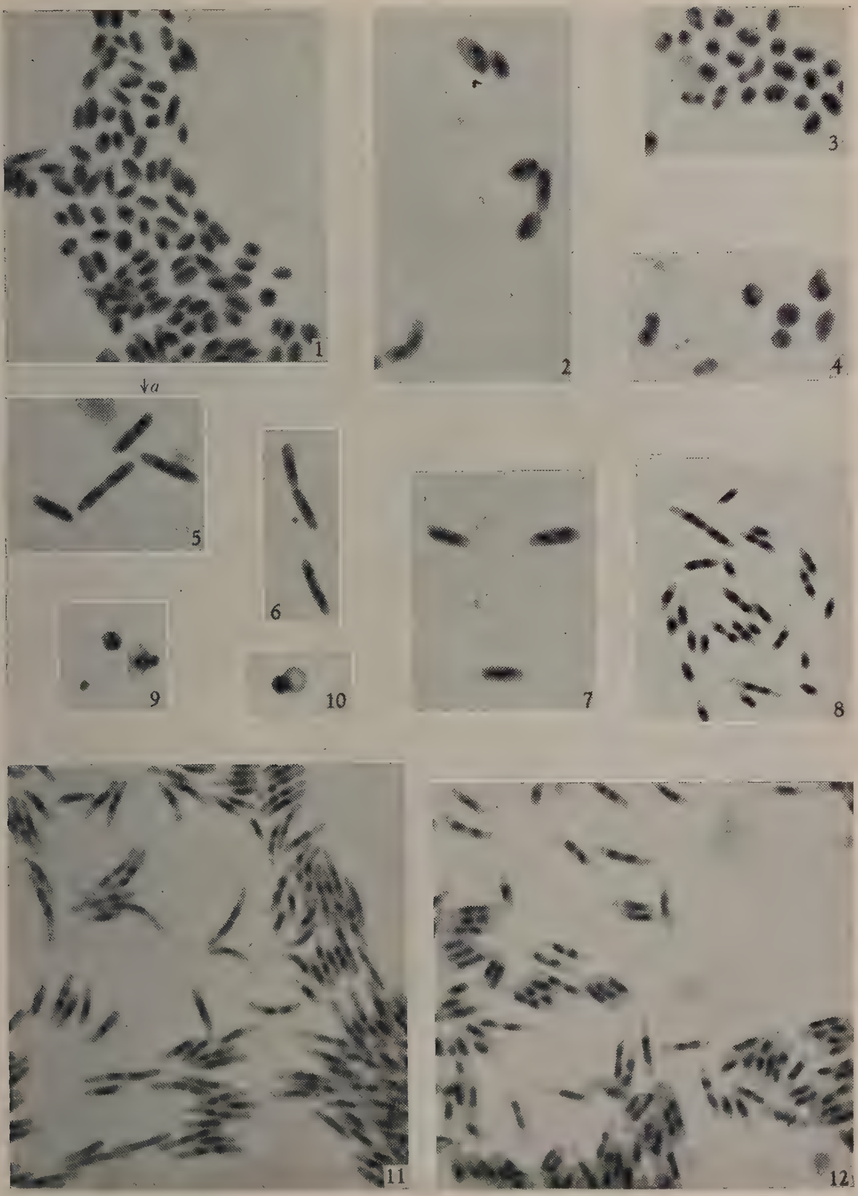
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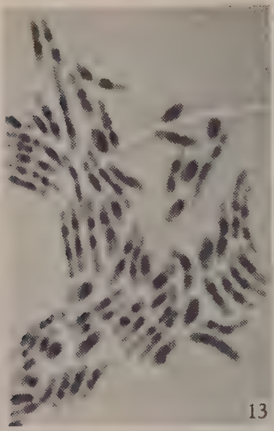
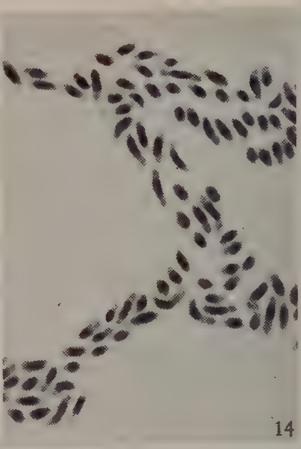
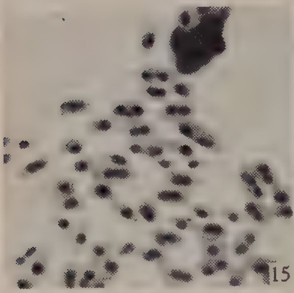
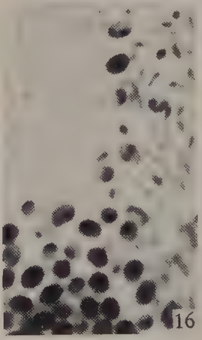
Figs. 1–12

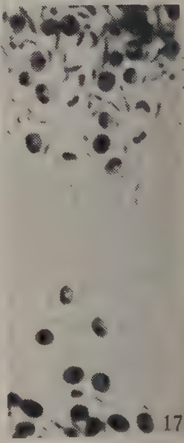
E. KLIENEBERGER-NOBEL—A CYTOLOGICAL STUDY OF MYXOCOCCI. PLATE 1

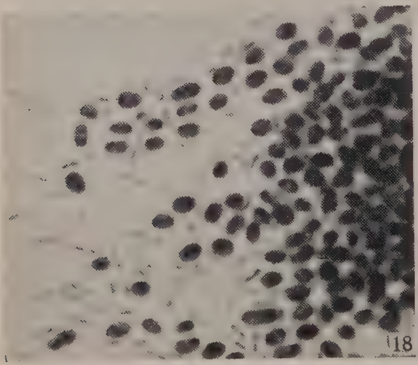


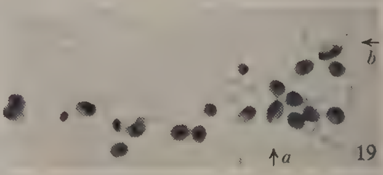












Figs. 13–19

E. Klieneberger-Nobel—A cytological study of myxococci. Plate 2

Differentiation of the Vegetative and Sporogenous Phases of the Actinomycetes

1. The Lipid Nature of the Outer Wall of the Aerial Mycelium

By DAGNY ERIKSON

Rothamsted Experimental Station, Harpenden, Herts

SUMMARY: The characteristic dry powdery appearance of the aerial mycelium of actinomycetes and the difficulty of wetting the constituent spores appear to be due to lipid substances in their outer walls. These substances are removed by fat solvents, and wetting agents, destroyed by alkali and are probably glyceride in nature. A staining with Sudan IV in ethanol clearly distinguishes the lipid-containing aerial mycelium from the vegetative mycelium.

The somewhat greater diameter of the aerial spore-bearing filaments of the ordinary aerobic actinomycete (the *Streptomyces* of Waksman, Group I *Cohnistreptothrix* of Ørskov) as compared with that of the vegetative portion of the mycelium has long been recognized (Ørskov, 1923). The marked resistance of the aerial spores to desiccation was noted by Acosta (1895) and Berestneff (1907) who established viability after 9 and 10 years' drying, respectively. More recently von Plotho (1940), as the result of various microchemical and tinctorial tests, stated that 'die Actinomyceten ihre Sporenmembranen mit Substanzen ausrüsten, die den Hyphenmembranen fehlen', but drew no definite conclusions as to the nature of these substances. In current reports on the production of antibiotic agents from stationary liquid cultures of the actinomycetes, there is frequent mention of the need for optimum yield of active substance, of a good surface growth (bearing aerial mycelium). The use of mass transfers of spores to obtain an early surface growth, and the failure to do so with an inoculum of mainly vegetative submerged colonies, are well known to all workers in this field. Nevertheless, the cause of the flotation of the spores and the sinking of the main mycelium has not hitherto been investigated. That the spores are strongly hydrophobic is evident from the common observation that a drop of water added to an old plate bearing a continuous sporing surface growth can be rolled round the dish without losing its shape, acquiring only an external coating of spores. This phenomenon did not escape the acute observation of Ørskov (1923), who recommended adding a drop of ethanol under the coverslip when examining colonies with an aerial mycelium 'since the aerial mycelium, which partly shuns water, will by this means be drawn down' (p. 39). An attempt has been made to relate this hydrophobe property to the lipid nature of the outer layer of the aerial filaments.

Organisms tested

Two characteristically pigmented varieties were extensively tested: (1) a member of the *Actinomyces coelicolor* species-group with its insoluble red, soluble blue, indicator pigment, having spirally coiled aerial hyphae with oval to spherical spores on most common media; (2) a member of the *A. griseofulvus* species-group, producing no soluble pigment, with fawn to greenish straight aerial hyphae and cylindrical spores. Both were isolated from soil.

In addition some 100 strains picked haphazardly from routine soil platings were tested with fat stains. They represented a considerable number of varieties, including several members of the chromogenous *A. albus* type producing a melanin on protein-containing media, *A. flavus, aureus, flavovirens, cellulosae*, etc. No attempt was made to give specific names to all the strains. The random method of selection ensured a varied sample of the soil population.

Two or three common penicillia and aspergilli, and some saprophytic strains of mycobacteria, sarcinae, streptococci, and coliform bacteria were used for comparison.

Fat stains

(*a*) *Sudan IV* (see Conn, *Biological Stains*, 1946). None of the methods commonly employed for demonstrating fatty substances *within* cells was suitable for this external sheath of fatty material. As already noted by Lewis (1941) dried fixed films are very unsatisfactory for the general run of fat-containing bacteria. The usual solvents—mixtures of ethanol and acetone—are unstable and precipitate the dye overmuch on the slide, though the technique of Burdon, Stokes & Kimbrough (1942), where an emulsion of cells is made in the solution of the dye (in their case Sudan black B) and a loopful rapidly spread with a circular motion on the slide, ensures that the precipitated particles are deposited at the periphery of the drop. Using this method with a 70% solution of Sudan IV in ethanol, a pink colour was achieved around clumps of 4-month-old spores; 3-day-old spores of the same strain did not stain; *Mycobacterium phlei*, in fairly dense masses, and a suspension of finely ground beeswax were positive; *Bacterium coli* and *Chromobacterium prodigiosum* were negative. But in all cases the pink stain was dull and faint compared with the brilliant red given by a drop of cotton-seed oil. *Iso*propanol, as recommended by Lillie (1944), improved the intensity and penetration of the stain, but the best results, with no precipitation of the dye, were obtained with *n*-butanol.

The following technique was elaborated. Actinomycetes are grown on the surface of sterile cellophan over agar of different compositions. Portions of the growth are removed entire by cutting sections of the cellophan at varying intervals. It is possible thus to obtain the whole of the substratum mycelium of a colony, which otherwise would be adherent to the agar, together with the delicately attached aerial mycelium *in situ*, and to preserve both intact on the cellophan throughout the staining operations.

A 70% butanol staining solution is prepared as follows: A, stock saturated solution; add 0·5 g. Sudan IV (B.D.H. 627701/44083) to 25 ml. *n*-butanol; boil (117°), cool and filter. B, mix 4·5 parts *n*-butanol with 5·5 parts by volume of ethanol. For the staining solution, add 7 volumes of A to 9 volumes of B and filter. This solution remains stable for months.

The cellophan bearing the growth is stained for 30 min., dipped for a few seconds into 70% ethanol to remove excess stain, washed in water, and mounted either in water for immediate examination or permanently in glycerol jelly.

In all strains tested the vegetative substratum mycelium remained entirely colourless, while the aerial filaments even before division into conidia showed varying degrees of external staining. Day-old, minute branches can be distinguished by the incrustation of red granules. Mature spores in long chains are evenly stained round each member (cf. Pl. 1, fig. 1). Precise definition is obtained only if the growth is not too dense (growth 5–10 days old, according to the nature of the medium, being suitable); the individual filaments of thick tufts of sporulating branches, characteristically situated on the crown of an older colony, tend to be stuck together with the dye, giving a blurred though abundantly stained picture. On the other hand, isolated spores mechanically disrupted from the branches are often unstained. Nevertheless, the validity of the technique has been generally confirmed by sampling aerial mycelium from various slope cultures on different media, spreading it out on cellophan, and staining as above, whereby the majority of the spores still adhering in chains of varying length will take the stain. Surface sporing colonies on liquid media require more care in handling and must be dried before the staining schedule is applied, but a proportion give positive results. Coverslips pressed on soil and sand cultures for a few days, then fixed in *n*-butanol and stained, in some cases, retain conidial branches in a sufficiently undamaged state to take the Sudan stain (Pl. 1, fig. 2).

The composition of the medium, therefore, appears to have no influence upon the production of the lipid substance or substances in the walls of the aerial mycelium. The point was further tested by growing the two chief test organisms on cellophan strips over a considerable variety of synthetic agars suitable for actinomycetes. The same differential staining of aerial and substratum mycelium was found, although naturally more vivid and consistent pictures were obtained on media such as starch tryptone agar which enhance sporulation.

As long as the aerial branches remain unbruised and unwetted, there is little diminution in staining capacity with ageing of the culture. Portions of growth on cellophan removed from the agar and kept in the dry state for over a year, and the surface growth on soil plate cultures allowed to dry out over the period of one year, have retained their staining properties.

No positive results have been obtained by any method with the bottom vegetative growths of actinomycetes in liquid cultures; with streptococci, a chain of which resembles in gross appearance an actinomycetal chain of conidia; or with any of the ordinary rod-shaped bacteria, cocci, or sarcinae tested. The dye aggregates loosely round sporing heads of aspergilli and penicillia, which, like those of actinomycetes, are also difficult to wet. Partial staining of the lipids in the acid-fast mycobacteria, which has frequently been described, was confirmed.

(*b*) *Osmic acid.* The walls of the spores but not the membranes of the vegetative hyphae were stained a characteristic yellow-brown when specimens were inverted for 2–4 days over a cell containing a drop of 2 % osmic acid in a moist chamber.

(*c*) *Oil blue N.A.* This dye (1, 4-*bis*-amylaminoanthraquinone; Calco., American Cyanamid Co.), which is used for staining rubber as well as fatty substances, acts rapidly either in the 70 % *n*-butanol solution or in 60 % *iso*propanol (cf. Lillie, 1945), producing in 5–10 min. a blue aerial and a colourless substratum mycelium, which after washing are dramatically distinguishable to the naked eye in the maturer colonies. Microscopically the picture is more blurred by dye precipitates than in comparable Sudan IV-stained material. A lactophenol solution prepared according to Wittenberger (1944) is stable, and if allowed to act for 24–48 hr., the material being subsequently washed in 25 % lactic acid, gives a more even stain.

Extraction with fat solvents

Portions of 7-day-old growth on cellophan over Czapek agar were extracted in small covered containers at 33° for 24 hr. with the following fat solvents: acetone, ether, chloroform, xylene, benzene, ethanol, *n*-propanol, *iso*propanol, *tert.*-butanol, and *n*-butanol. They were then dried and stained by Sudan IV. In all cases excepting the *n*-butanol treated specimen, the staining of the aerial mycelium was very much less than in untreated material. The *n*-butanol extracted growth, though brilliantly red to the naked eye, showed on microscopical examination a rather fuzzy outline as compared with the control, probably due to the enhanced adsorption of the dye. Acetone, ether, and benzene removed almost all the stainable material round the spores, which were a faint yellowish pink. Chloroform and xylene were nearly as effective. Ethanol yielded a poorly stained picture with a considerable amount of dye precipitate. Somewhat pinker spores were left in the material treated by the propanols and by the *tert.*-butanol. In all instances the spore membrane, even if only faintly coloured, appeared to be thicker than the cell walls of the substratum mycelium.

The efficacy of the acetone and ether treatments suggests (*a*) that the bulk at least of the fatty material is not phospholipin in nature, and (*b*) that it is not very firmly bound in the cell wall. An attempt was therefore made to extract the substance by a simple modification of Anderson's (1932) methods for the lipids of tubercle bacilli. The 5-day-old, abundantly sporing cellophan growth of fifty plates was submitted to ether-ethanol-chloroform extraction in the cold. The final chloroform extract on evaporation *in vacuo* yielded a minute quantity of an orange-coloured, waxy, semi-fluid substance. The amount was too small for detailed tests, though it proved to be glyceride in nature. Cotton-wool fibres rubbed into the substance retained the wax in small agglomerates which coloured a brilliant red with Sudan IV, and which darkened on exposure to osmic acid. It is, of course, impossible to prove that part at least of this substance was not extracted from the interior of the cells, but it is worth noting that Lieske (1921) found that with most actinomycetes the old dried cultures, which would probably be abundantly sporing, yielded a small quantity of ethereal extract 'der scheinbar auf Fette zurückzuführen' (p. 88).

Saponification

Portions of 7-day-old growth on cellophan placed in ethanolic KOH in covered glass vessels lost almost every vestige of stainable material within 3 hr. at 33°. Similar material sealed in a cell with a few drops of ammonia and KOH according to the method of Molisch (1923) yielded after 2 days some crystals, presumably of soap, as well as amorphous protrusions round the aerial filaments. Other portions of cellophan growth placed in 10% ammonia were found to show a yellowing and apparent thickening of the spore membrane, frequently in bipolar fashion, which was visible on the 2nd day of treatment, obvious from the 6–12th days, thereafter degenerating into irregular swellings.

Action of wetting agents

Large single colonies, one month old, were treated for 3 days in 1% soap solution, and also in three different commercial detergents. When washed, dried, and stained with oil blue N.A., they were conspicuously unstained in contrast to colonies taken from the same plate, which were either untreated or dipped in *n*-butanol. Three-day-old growth of the same strain, when immersed in one of the detergents (solution A, a sulphonated long-chain ester), was wetted almost instantaneously and sank to the bottom of the container in less than 5 min. At this period it still took up the fat stains, but less vividly than the control. Untreated growth which has already taken the stain is not decolorized by immersion for prolonged periods in the wetting agents. Untreated spores ride in a layer on the surface of an aqueous suspension and extend in a film up the walls of the tube, the greater part remaining there even after 5–10 min. centrifugation at 10,000 r.p.m. This property is to a very considerable extent lost if the spores are first washed off from the growth in detergent A instead of in water; the majority go into suspension and deposit on standing.

This work was carried out by the author as a member of the scientific staff of the Agricultural Research Council.

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EXPLANATION OF PLATE

Fig. 1. Mature spores stained with Sudan IV, substratum mycelium colourless; cellophan growth on agar 8 days.

Fig. 2. Aerial mycelium not divided into spores, stained with Sudan IV, substratum mycelium colourless; impression preparation from soil culture 4 days.

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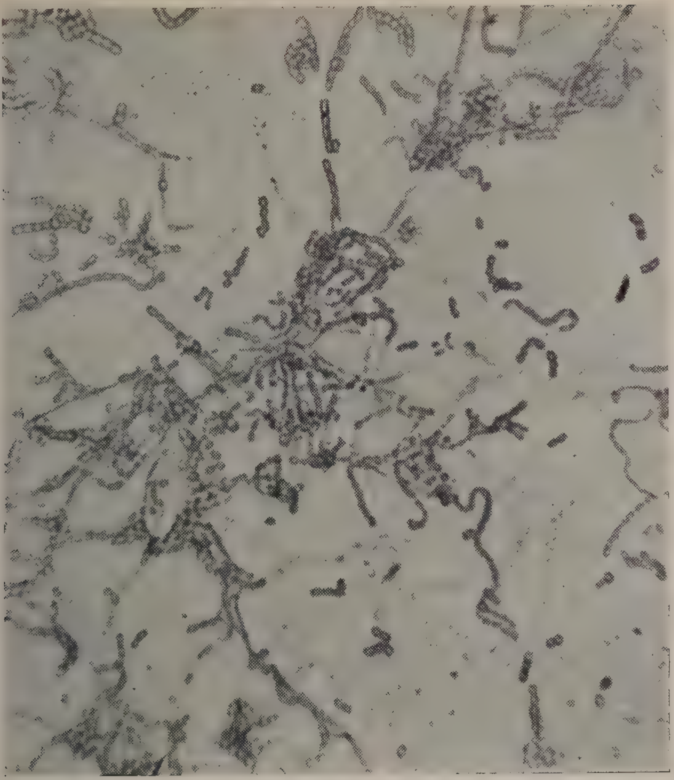

Fig. 1

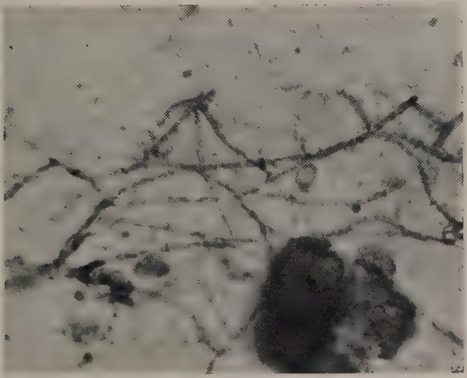

Fig. 2

D. Erikson—Lipids in actinomycetes. Plate 1

Differentiation of the Vegetative and Sporogenous Phases of the Actinomycetes

2. Factors affecting the Development of the Aerial Mycelium

By DAGNY ERIKSON

Rothamsted Experimental Station, Harpenden, Herts

SUMMARY: When first isolated on soil extract agar, soil actinomycetes consistently produce aerial spores in surface colonies. They retain this property when maintained in sterile soil, or when grown on washed suspensions of common soil bacteria, living or dead, in a water agar medium. In soil, when the composition, moisture content and temperature are kept constant, the initial stimulus towards the production of aerial mycelium is free access of air: the quantity and nature, vegetative or sporogenous, of the inoculum, and, within a broad range, the pH of the soil are of minor importance. Once growth is established, the next most important factor stimulating sporulation in the soil is also physical, namely dehydration. In natural and sterilized soils of different origins, and in a 'synthetic' soil containing 250 p.p.m. of nitrogen as nitrate, the modes of growth of different actinomycetes strains are similar, and generally uncharacteristic of their species.

The stress laid upon the variability of the actinomycetes in general, as reflected in their remarkable responsiveness to changes in media, in the extensive studies of Waksman (1919) and of Lieske (1921), has been echoed by most succeeding workers in the field. The two outstanding gross variations are in pigment production and the appearance of the aerial, spore-bearing apparatus. Soluble pigment production, whether of the yellow-brown (Plotho, 1940) or of the red-blue (Oxford, 1946) type has been shown in mass cultures to depend on the pH reached in the varying stages of metabolism of different nutrient substances. The striking difference between the smooth, cartilaginous, dense-textured colonies composed of vegetative hyphae only, and the 'powdered' growth characteristic of an abundant production of aerial mycelium arising from ramified substratum filaments (see Pl. 1, figs. 1*a*,*b*), is not so readily attributable to single factors. Nevertheless, the ability of the organism to reproduce itself by means of spores is biologically one of its most important properties. This paper reports an investigation of some of the factors which affect this property.

Composition of the medium

The effect of readily available nitrogen. Afanasiev (1937), working with parasitic and saprophytic strains of *Actinomyces scabies*, attempted to find the C : N ratio which favoured the development of the vegetative growth only. In a synthetic solid medium containing glucose as the main source of carbon, a high proportion of nitrogen, supplied either as KNO_3 or asparagine, partly suppressed the development of aerial mycelium. The organic nitrogen compound was particularly effective, a phenomenon noted also by Erikson (1935) in a group of actinomycetes isolated mainly from human pathological material. In twenty of twenty-six strains no aerial mycelium developed on complex nitrogenous media such as inspissated serum, blood agar or Dorset egg, and only very occasionally a sparse aerial mycelium developed on heart broth and glucose heart broth agars. Aerial growth was obtained only on simple synthetic media like Czapek's sucrose or glycerol or Ca malate agars, or starvation substrates like Ørskov's tap-water agar; and with some four or five strains not even on these. In the present work fifty out of seventy-two strains of saprophytes selected at random from routine soil platings developed the exclusively vegetative growth of the kind pictured in Pl. 1, fig. 1*a* on a peptone Lemco agar with the addition of 0·5 % lecithin, although all except five of the fifty produced aerial growth on the same peptone agar without the lecithin; and all yielded an abundant development aerial growth like that in Pl. 1, fig. 1*b*, when starch was substituted for lecithin. On Czapek's sucrose nitrate agar almost every one produced an aerial growth which was abundant in comparison with the thinly spreading substratum mycelium.

The exclusively vegetative growth formed on the complex phospholipin-containing media is almost always considerably bulkier than that produced on the simpler organic or inorganic substrates. It is also less viable. This was generally true of about 300 strains of actinomycetes belonging to Ørskov's Group I. Readily available nitrogen in excess, therefore, favours the multiplication of the vegetative hyphae at the expense of the biologically more economical and reproductively more efficient aerial spores.

Bacteria as a source of nitrogen. This is not the case with nitrogen in the form of dense suspensions of washed bacteria, living or heat-killed, in tap-water agar. The vegetative growth is not enhanced, not even for those strains of actinomycetes with considerable bacteriolytic activity, that produce a clear area in the vicinity of their growth. All of 124 strains, which when tested against fifteen varieties of soil bacteria, were lytic in varying degrees and grew on the bacterial agar with only a moderate substratum mycelium that soon gave rise to aerial sporogenous hyphae. Both phases of growth, however, were similar in morphology and bulk to those of the same strains grown on bacteria insusceptible to lysis. It would appear, therefore, that the amount of nitrogen rendered accessible to the growing actinomycete by the lysis of the bacteria that it induces must be small and little more than that found in the autolysates normally present in a suspension of insusceptible bacteria. In this connexion it is noteworthy that a definite stimulation of growth is observed when bacterial cells are added to a water-agar medium, but that no better growth of the actinomycete occurs where the bacteria are visibly lysed than where they are not.

Soil extract. When soil dilutions are plated directly on soil-extract agar the actinomycetes appearing as surface colonies invariably develop some aerial mycelium. This proved to be the case with several hundred strains isolated from differently treated soils, and emphasizes the fact that freshly isolated actinomycetes grown on a medium whose nitrogen content approximates to that of their natural habitat commonly display their normal powers of reproduction by aerial spores.

Influence of penultimate medium

Jones (1946) described the cultivation of actinomycetes in moist sterile soil as a means of maintaining their native properties. The method has been effective in the present work, though it was found unnecessary to keep the soil moist. Several strains were sown into sterile soil, allowed to grow, and left to dry in the medium for periods exceeding one year. All were subsequently recovered in their original form by plating on 'synthetic' agar. All such cultures developed normal aerial mycelium on starch tryptone, Czapek and other simple media, whereas subcultures of the same strains, kept in the vegetative phase in nutrient glucose broth for prolonged periods and then plated on similar media, occasionally produced variant sectors or colonies that for at least three or four generations were devoid of aerial growth. It is very probable that the sparse sporulation found for most of the twenty-six strains of pathological origin investigated by Erikson (1935) was in great part due to their continuous cultivation over a period of years on rich laboratory media.

Minimal requirements for growth

The identification of the substances in a medium which favour the development of aerial mycelium is difficult by reason of the simplicity of media that support the growth of the saprophytic soil actinomycetes. The trace elements present in tap water and the impurities in commercial agar make a medium which, since its introduction by Ørskov (1923), has been found sufficient for a thin substratum and aerial growth of many strains. As the following experiment shows, very little need be added to a purified agar to obtain similar growth.

To a well-washed agar made up in distilled water were added varying amounts of sterile glucose in distilled water. Plates were made and seeded with a row of droplets of a spore suspension from each of the strains tested. After 3 days' incubation at 33° two of seven strains produced aerial mycelium in the presence of 0·0625 and 0·0312 % glucose, and vegetative growth with only 0·0019 % glucose. After a fortnight, fair growth developed throughout the series with 0·0312 % glucose, while all strains yielded a very thin growth with a delicate but perceptible aerial mycelium, quite visible to the naked eye in the presence of 0·0019 % glucose.

The limiting concentration of glucose for growth in liquid media also was 0·002 %, using sodium nitrate or ammonium phosphate as the nitrogen source. From eight and ten similar strains, minute colonies were produced as bottom and surface growth, the surface colonies all giving rise to aerial hyphae after 2 days. With 0·001 % glucose, only two or three colonies were produced from two strains. The same liquid media without added nitrogen did not support visible growth.

Nature of inoculum

Millard & Burr (1926) pointed out that to secure early surface growth and sporulation on liquid cultures, inoculation of spores is essential. The lipid nature of the spore membrane (Erikson, 1947) enables it to remain floating and to germinate in the most favourable conditions for aerobic growth; vegetative growth sinks. The diagnostic criterion of primary vegetative bottom growth in liquid media, which characterizes Group I actinomycetes, enunciated by

Ørskov and confirmed for the most part by Erikson (1935), is by no means universally applicable and is indeed limited to subcultures (*a*) from substratum mycelium on agar cultures; (*b*) from bottom-growth colonies in liquid cultures; (*c*) from good sporulating growth to media in which there is a sufficient concentration of surface-active substances to cause the spores to sink; and (*d*) from poorly sporing material in which the few spores remain attached to the vegetative growth and fall with it to the bottom. Unless spores are made to sink, surface growth may occur. In fact, Ørskov (p. 47) ensures that the spores do not float by drying them first on sterile filter-paper, and states that if the inoculated spores remain on the surface, they quickly form a mycelium.

The growth phase of the inoculum used for sowing liquid cultures may be of great practical importance. For example, an asporogenous variant of *A. griseus*, producing only submerged vegetative growth in stationary flasks was found by Schatz & Waksman (1945) to produce no streptomycin. Furthermore, when the substratum mycelium only of active, sporing strains was inoculated, a similar type of submerged growth resulted, devoid of antibiotic substance.

Factors influencing growth in natural and sterile soils

Kubiena & Renn (1935), examining undisturbed, naturally developed soils in New Jersey, U.S.A., with the aid of a special vertically illuminated microscope, found actinomycetes growing particularly well in the soil spaces opening to the surface. All their illustrations show the same type of growth, predominately aerial tufts of hyphae in more or less compact colonies with long twisted strands bridging the gulfs between soil crumbs. Similar growth was obtained when soils from different Rothamsted plots, treated or untreated, from Marlborough chalk-down arable or from Thames-side pasture lands were left undisturbed in sterile Petri dishes. In such soils a surface growth of actinomycetes is frequently visible to the naked eye after 3 months or more as a faint greyish veil round the rim of the dish, where aeration is greatest and there is an accumulation of nutrients consequent upon the evaporation of water. This growth is almost entirely aerial, and uniformly colourless, although the cultivation of samples on artificial media yields various chromogenic strains.

Growth on buried coverslips. Coverslips buried beneath the surface of natural soils that still retain their natural moisture content become covered with growth, commonly in the form of loose, straggling, vegetative filaments, from which occasional sporing branches may arise wherever there is a minute air-space (Pl. 2). Lutman's (1945) criticism of the very similar findings obtained by Starkey (1938) as the result of using Cholodny slides—that the slide disturbs the soil-particle arrangement and introduces unnatural air pockets—is not valid in the present instance. For similar results were obtained by burying fragments of coverslips about one or two microscope fields in diameter, which can justifiably be claimed to cause no greater disturbance than many of the foreign particles commonly present in soils.

Sterilization. Sterilization of the soil does not affect the mode, abundance or the appearance of the growth of various actinomycetes. Sterile soil moistened with sterile distilled water, and evenly inoculated with a watery suspension of washed, lightly ground, vegetative mycelium derived (*a*) from bottom growth in liquid cultures, (*b*) from non-sporing surface growth on rich agar media, developed in 6 days at 25° a copious aerial mycelium covering almost every surface crumb. As good, but no better, results were obtained with an aqueous suspension of spores of the same strain. Successful inoculations were also made with the smallest possible soil crumb which could be lifted on a needle from an already grown plate, placed in the centre of a fresh plate of sterile dry soil and subsequently moistened by a fine spray of water.

Site of inoculations. The importance of the site of inoculation for growth in undisturbed soils is shown by the following experiment.

Discrete colonies, approximately 3 mm. in diameter, of *Actinomyces* strain 6513 Al, 20 days old, with well-developed aerial mycelium, which had been grown on cellophan over a synthetic agar, were cut out and inoculated (*a*) in the centre of the bottom of the dish, cellophan downwards, and the soil carefully replaced over the colony, (*b*) in the centre of the surface of the soil, cellophan uppermost, and (*c*) dispersed in sterile water and thoroughly mixed with the soil. In (*a*) and (*b*) 25 % water by weight was added to the soil (Barnfield, unmanured) before inoculation. The three tests were repeated using as inocula large 2 × 1 in. strips of cellophan bearing uniform growth of the same strain. After 2 days' incubation at 25° samples were taken from the soil plates with a sterile cork-borer, both from the immediate vicinity of the centre and from the periphery of the plate, diluted 1000-fold, and plated.

Table 1. *Showing the influence of site of inoculation on spread of actinomycetes in plates of sterile soil*

	No. of colonies developing from the	
Mode of inoculation	Central sample	Peripheral sample
One colony, buried	0	0
One colony, on top	500	0
One colony, dispersed	P	P
Strip, buried	70	0
Strip, on top	300	0
Strip, dispersed	P	P

P = profuse growth.

Table 1 shows that with a surface inoculum growth in the immediate vicinity of the site of inoculation is abundant and unrelated to the size of the inoculum (one colony or a large strip); with a buried inoculum of either size growth is poor; and only when the inoculum is dispersed does growth extend to the periphery of the plate.

Coverslips buried halfway between centre and periphery of replicate plates, when examined a fortnight later, showed growth of the typical vegetative filamentous pattern with the occasional sporing branches portrayed in Pl. 1, fig. 2, in every instance when the inoculum was dispersed, in three of five cases with the strip on top, in two of five cases with one colony on top, in two of five with the buried strip, and in one of five with the buried colony. At this time a surface development of aerial mycelium could be detected microscopically in all soil plates, and was visible to the naked eye wherever the inoculum had been introduced from above.

Thus, when the composition of the soil, moisture content, and temperature are kept constant, the initial stimulus towards the production of sporogenous hyphae is free access of air.

Size of soil crumb. To test the influence of the size of soil crumb and corresponding differences in air-space, allotment soil was graded through three sieves of 0–1, 1–3, and 3–4 mm. mesh and a series of plates made up with different moisture contents obtained by spraying the water on with an atomizer, smoothing the surface as far as possible, and then autoclaving. The moisture content after sterilization was determined. A central plug was removed with a sterile cork-borer from each plate, and varying amounts from 0·01 to 0·5 ml. of an aqueous suspension of spores of *Actinomyces* strain '*g*' were introduced into the hole, the soil being carefully restored and pressed flat. The plates were incubated for 15 days at 25°. In all dense aerial growth was macroscopically visible in the central area extending from the site of inoculation irregularly for 0·5–5·0 cm. No correlation could be observed between extent of growth and the varying moisture contents (15–25 %) or the size of inocula. But whereas with the large crumb size (3–4 mm.) a delicate greyish veil of diffuse aerial growth could be seen almost all over the plate (see Pl. 2, fig. 3), with the finer crumbed soils (especially 0–1 mm.) growth was limited outside the central zone to discrete colony masses which sometimes reached as much as 10 mm. in diameter and which resembled growth on solid artificial media (see Pl. 2, fig. 4). A similar set of plates were stacked in piles in a large glass container with a little water at the bottom, and held in a greenhouse subject to fluctuating day and night temperatures. Here, after the same period, growth was of the types described above, but noticeably less in extent in every case. It was particularly noteworthy that the top plate of each pile showed no visible growth, an effect probably due to the adverse effect of bright sunlight on the development of aerial mycelium already noted by Ørskov (1923). In this country the ordinary diffuse daylight reaching a laboratory bench in front of a window has not been found to have any such effect; the greenhouse experiment, however, was carried out during a bright spell in the spring of 1945.

Hydrogen-ion concentration. All the sterile soils supporting growth were maintained at a pH of about 7·0. The natural soils ranged from pH = 4·2 to 8·7. It has already been mentioned that none of the naturally occurring or inoculated actinomycetes growing in the different specimens of soils examined produced pigmented growth. An attempt was made to see whether pigment production could be induced by varying the pH. A vigorous strain of *A. coelicolor* was sown in massive doses on the smoothed surface of sterile soil plates adjusted to pH = 4·3 with acetic acid, and to pH 8·6 with sodium bicarbonate. Growth was fair, but identical in both cases, colourless aerial mycelium appearing in the uncharacteristic cottony tufts observed throughout this work, with a sparse development of undifferentiated vegetative filaments that became increasingly fragmented as the soil dried. However, the spirals characteristic of the aerial hyphae of this strain could be seen in many places irrespective of pH of soil.

Growth in synthetic soils

A 'synthetic' substrate physically similar to soil has been described by several investigators. The sand-bentonite mixture of Madhok (1937) has been found most satisfactory for growth experiments.

It was prepared in the proportion of 47·5 g. washed, ignited quartz sand to 2·5 g. bentonite per plate. To this was added cellulose in the form of shredded Swedish filter paper or cotton-wool, and 0·05 g. of the dried insoluble residue left after extracting 5 g. of dried autolysed yeast three times in 100 ml. distilled water. The plates were dry-sterilized. An artificial soil solution based on the average figures quoted by Russell (1927) was made up as follows: $Ca(NO_3)_2$...0·33 g., $CaSO_4$...0·8 g., $MgSO_4, 7H_2O$...0·7 g., K_2SO_4...0·025 g., K_2HPO_4...0·005 g., $NaHCO_3$...0·2 g., water to 1 litre. This gives total solids, in p.p.m., of Ca 310, SO_4 850, Mg 70, Na 55, K 13, HCO_3 140, PO_4 3, NO_3 250: total = 1691. This solution, sterilized separately, was used as the suspending fluid for the inoculum of spores and added to the sand plates.

Growth was visible in 5 days at 25° in plates containing 8–24 % by weight of the solution and, although sparse in the lower concentrations, was of the same type throughout—minute tufts of colourless aerial mycelium, which in many strains were attached directly to the quartz particles (see Pl. 2, fig. 5) and not to the filter paper or cotton-wool fibres. On buried coverslips fragments of the initial vegetative mycelium could be discerned, but often with difficulty owing to the obscuring effect of the colloidal bentonite. Enhanced growth was obtained with extra nutrients, but the mode of growth was the same in all cases—a sparse development of vegetative mycelium followed by an early and relatively abundant production of aerial sporogenous hyphae on the surface and in the air-spaces below the surface. The type of growth was substantially similar to that in natural soils, native or sterile.

DISCUSSION

Any ready evaluation of the factors stimulating aerial growth of soil actinomycetes can only be roughly qualitative because of their ability to develop on a minimal supply of nutrients. Thus, 0·002 % glucose in a washed, but not necessarily nutrient-free, agar supplied the requirements for complete growth. The provision of nitrogen in excess, however, particularly in the form of phospholipins in artificial media, favours the growth of the vegetative at the expense of the aerial mycelium in a large number of soil actinomycetes. This effect of rich media can to some extent be imposed on the organisms, for strains maintained in the vegetative phase in nutrient glucose broth tend to produce sporeless variants when subcultured on to solid media.

In liquid cultures the primary factor ensuring sporulation is the flotation of an inoculum of spores upon the surface, made possible by reason of the lipid nature of their external surface.

This work was carried out by the author as a member of the scientific staff of the Agricultural Research Council.

I wish to thank Dr H. G. Thornton, F.R.S., for helpful suggestions and criticism, and, together with Mr Stansfield, for taking the photomicrographs; Mr A. V. Garcia for many cultures and for showing me the results of his large-scale isolations from different soils; Dr A. R. Martin (Imperial Chemical Industries Ltd.) for a supply of detergents; Miss Mabel Dunkley and Miss Mary Williams for technical assistance.

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EXPLANATION OF PLATES

Plate 1

Fig. 1*a*. Colonies of actinomycete '*g*' on peptone-lecithin medium, showing only vegetative mycelium—14 days.

Fig. 1*b*. Actinomycete '*g*'—single colonies, on starch tryptone agar, 2 weeks, showing abundant aerial mycelium.

Fig. 2. Coverslip buried in garden soil 9 days, showing long straggling filaments of vegetative mycelium of wild *Actinomyces*—fixed osmic acid, stained haematoxylin by Dr B. N. Singh.

Plate 2

Fig. 3. Soil, 3–4 mm. crumb, 1 month, showing faint greyish veil of growth over soil crumbs.

Fig. 4. Soil, 0·1 mm. crumb, 1 month, showing macroscopic colonies.

Fig. 5. Pure culture of *A. coelicolor* in 'synthetic' soil—yeast residue, 2 weeks. Growth attached to quartz particle.

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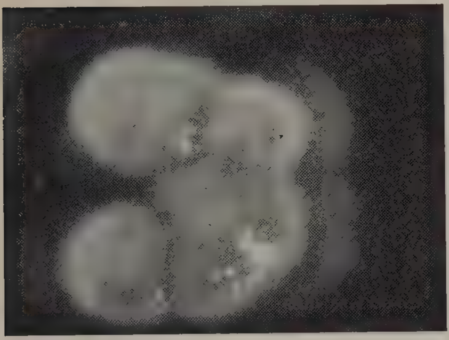

Fig. 1*a*

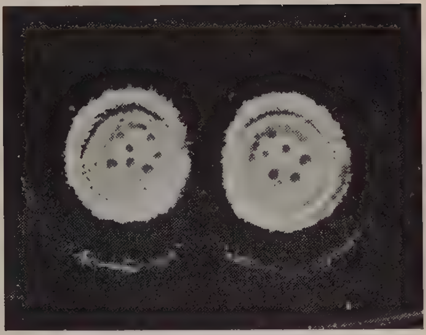

Fig. 1*b*

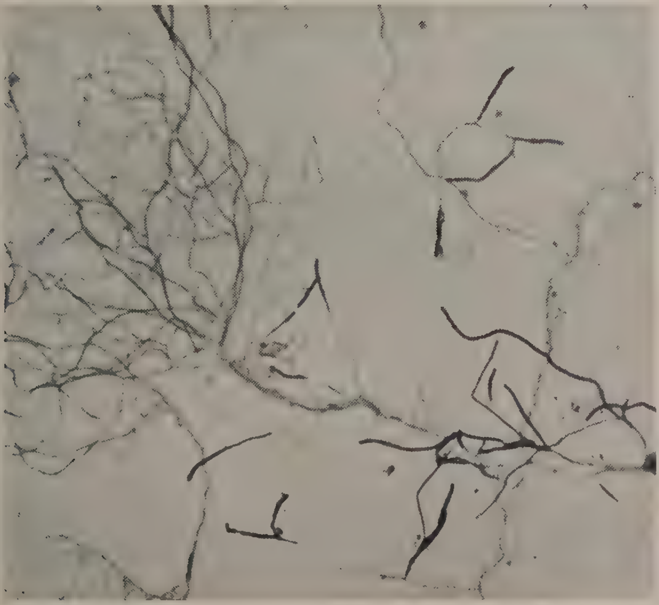

Fig. 2

D. Erikson—Development of aerial mycelia in actinomycetes. Plate 1

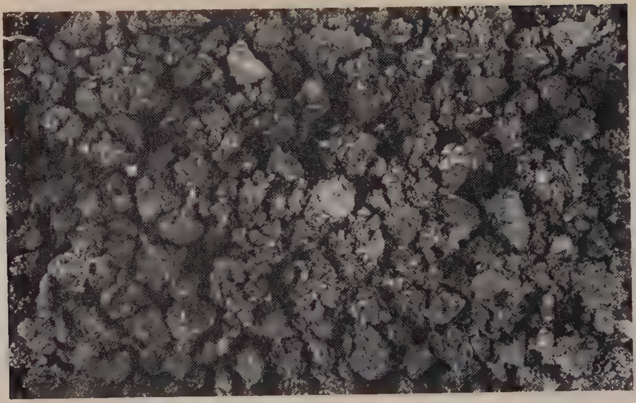

Fig. 3

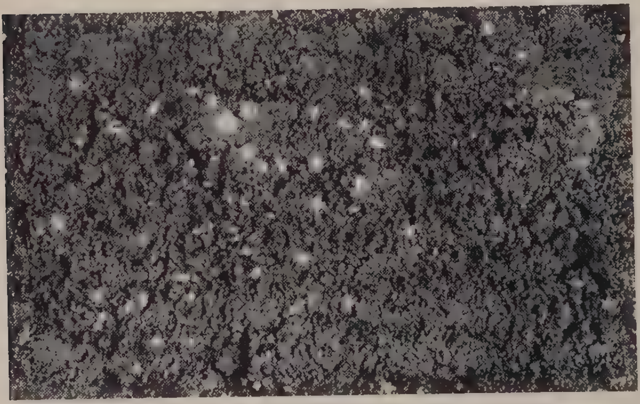

Fig. 4

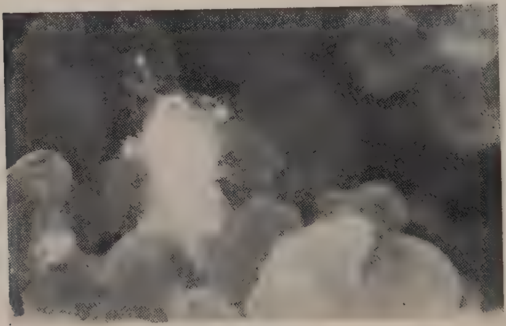

Fig. 5

D. Erikson—Development of aerial mycelia in actinomycetes. Plate 2

The Assimilation of Amino-acids by Bacteria

1. The Passage of certain Amino-acids across the Cell wall and their concentration in the internal environment of *Streptococcus faecalis*

By E. F. GALE

Medical Research Council Unit for Chemical Microbiology,
Biochemistry Department, Cambridge

SUMMARY: By a method for determining the free amino-acid content of bacterial cells, *Streptococcus faecalis* (Lancefield Group D) was found to have free lysine, glutamic acid, ornithine and histidine in its internal environment, the amount depending to a certain extent upon the growth medium. The internal lysine and glutamic acid are unable to diffuse out of the cells when these are suspended in water or suitable salt solutions.

When the cells are grown in a medium deficient in amino-acids, the internal environment is also deficient and those cells can be used to study the conditions governing the passage of certain amino-acids into the cell. Lysine enters the cell by diffusion, probably passing the cell wall in the isoelectric state. Glutamic acid, glutamine and histidine cannot pass across the cell wall unless energy is supplied by some exergonic metabolism such as the simultaneous fermentation of glucose.

Under equilibrium conditions the internal concentration of free lysine or glutamic acid within the cell is markedly greater than that outside; the lower the external concentration, the higher the concentration difference across the cell wall.

Glutamic acid inside the cell can pass out into the external environment if fermentation is taking place. The final equilibrium conditions are the same whether the glutamic acid is entering or leaving the cell. Lysine cannot diffuse freely out of the cell, but outward migration takes place if fermentation occurs simultaneously. The uptake of lysine by deficient cells is decreased by the presence of glucose and this effect is, in turn, partially abolished by the uptake of glutamic acid in the presence of glucose.

Recent studies of bacterial metabolism have thrown considerable light on the amino-acid requirements of bacteria, and of the mechanisms utilized for the breakdown of amino-acids. Generally speaking, the organisms appear to fall into two groups. Members of the first group, which includes many organisms loosely classified as Gram-negative, are able to synthesize most if not all of their amino-acid requirements and also to produce a variety of catabolic mechanisms. Members of the second group, which includes many Gram-positive organisms, are often able to synthesize but few of their amino-acid requirements and have very restricted catabolic activities towards amino-acids. Moreover, the growth processes of the second group (e.g. *Strep. haemolyticus*) are markedly more susceptible to interference by bacteriostatic agents such as penicillin, the sulphonamides, certain dyes, etc., than are those of the first group (e.g. *Bacterium coli*). There would appear to be some fundamental difference between the two groups the elucidation of which should add materially to our knowledge of bacterial growth processes and their inhibition by chemical means. It is possible that this difference lies somewhere in the synthesis of protein by the two groups, but little is known, as yet, of the biochemical changes that intervene between the synthesis of amino-acids and their eventual appearance as bacterial protein. This series of papers represents an attempt to investigate the processes involved in the assimilation of amino-acids by bacteria, the effect thereon of bacteriostatic agents, and the differences displayed by Gram-positive and Gram-negative organisms.

EXPERIMENTAL

Methods

Organism and growth media. The work described in this paper was carried out with a non-haemolytic Group D *Streptococcus* (N.C.T.C. no. 6782).

Two media were used for growth purposes:

Medium A consisted of a tryptic digest of casein + 0·1 % Marmite + 1·0 % glucose.

Medium B consisted of a simplified medium similar to that previously described (Gale, 1945*a*) with the difference that the Marmite was not hydrolysed and no cysteine was added.

For diffusion experiments, etc. the cells were suspended in a salt solution containing: 0·1 % KH_2PO_4; 0·33 % Na_2HPO_4; 0·1 % NaCl; 0·07 % $MgSO_4.7H_2O$; the pH was adjusted to 7·2. Dry weights of organism in cultures and washed suspensions were estimated turbidimetrically by the use of a Hilger absorptiometer previously calibrated against suspensions of the organism of known dry weight.

Estimation of amino-acids. The estimations of the amino-acids described in the paper were carried out manometrically by the use of specific amino-acid decarboxylase preparations (Gale, 1945*b*, 1946*a*). These preparations estimate the free unsubstituted 'natural' amino-acids only.

Preliminary work

In the course of some investigations into the amino-acid constitution of various bacteria, it seemed probable that the method of analysis of acid hydrolysates of thick washed suspensions might give inaccurate results if significant amounts of free amino-acid were carried down from the medium either on the surface of the washed organisms or in their internal environments. To test this possibility, a thick suspension of streptococcal cells, grown in medium A and washed twice in water, was divided into two equal parts. One was hydrolysed in *c.* 5N-HCl for 24 hr. and the HCl then removed *in vacuo*, and the other was shaken with glass beads on the shaker (Curran & Evans, 1942) for 7 hr. after which the viable count was reduced to 0·001 % of the initial value. The two preparations were then made up to the same volume and their free amino-acid composition determined. The results are shown in Table 1; 31·0 % of the total glutamic acid estimated in the acid hydrolysate was found in the free state in the disintegrated cell suspension; 25·5 % of the total lysine, 25·0 % of the total histidine and 92·1 % of the total ornithine were also estimated in the cell disintegrate. No free arginine or tyrosine was found in the disintegrate.

The free amino-acids of the cell disintegrate might arise in a number of ways. First, it is possible that the cell protein becomes broken down during the disintegration of the bacterial cells; if this were the case it is difficult to see why no free arginine or tyrosine should be found and repetition of the disintegration at 0° or in the presence of toluene or caprylic alcohol had no effect on the result. Secondly, the free amino-acid might be carried down from the medium absorbed on the surface of the organism. Thirdly, the amino-acids might exist in a free state in the internal environment of the cells. Using the decarboxylase method of estimating amino-acids it is possible to distinguish between these three possibilities. If a decarboxylase preparation is added to a suspension of bacterial cells the enzyme cannot penetrate the cell wall and any amino-acid estimated must therefore be present outside the cell. Fig. 1 shows the effect of

Table 1. *The amino-acid composition of* Strep. faecalis *cells*

Results expressed as amino-acid N as % total N.

Amino-acid	Acid hydrolysate (*a*)	Disintegrate (*b*)	Combined amino-acid (*a*−*b*)
l-(+)-Lysine	11·30	2·90	8·40
l-(+)-Arginine	5·19	0·10	5·09
l-(+)-Glutamic acid	5·60	1·74	3·86
l-(−)-Histidine	2·84	0·70	2·14
l-(+)-Ornithine	1·48	1·27	0·21
l-(−)-Tyrosine	0·81	Nil	0·81

adding glutamic acid decarboxylase from the side-bulb of a manometer to a thick suspension of washed streptococcal cells in M/5 acetate buffer pH 4·5 (Gale, 1945*b*). A small CO_2 output occurred, representing the decarboxylation of glutamic acid carried down with the cells during washing. The amount of this glutamic acid varied with the degree of washing of the cells prior to estimation. If an equivalent amount of the cell suspension is now disintegrated by shaking, the amount of glutamic acid estimated is greatly increased. The same result is obtained if the cell suspension is placed in boiling water for 10 min. before estimation. If the cells are disintegrated by mechanical means or disrupted by heat, glutamic acid is released in the free state and this free amino-acid must presumably be held within the internal environment of the cells prior to disruption. This is confirmed, as shown later (Gale & Taylor, 1946), by the fact that the same amount of free amino-acid is released from the cells if the permeability of the cell wall is altered by the action of detergent substances.

It is possible to estimate the free amino-acid content of the internal environment of the cells by carrying out decarboxylase estimations (1) on the intact cells, and (2) on disrupted cells, and so determining the amount of amino-acid released on disruption. Since placing the cell suspension in boiling water gives the same result as mechanical disintegration but in a much shorter time (10 min., instead of 6 hr.), the cells have usually been disrupted in this way in the work to be described. Fig. 1 gives representative results obtained with the glutamic acid decarboxylase and a thick suspension of *Strep. faecalis* cells. Using the other specific decarboxylase preparations, it was possible to show the existence of lysine, histidine and ornithine, in a free state in the internal environment of these cells. No free arginine or tyrosine was found probably because the organism possesses a very active arginine dihydrolase (Hills, 1940) and tyrosine decarboxylase (Gale, 1940) so that the growth medium contains no free arginine or tyrosine at the time of harvesting (Gale, 1945*a*). The action of arginine dihydrolase gives rise to the formation of ornithine in the medium and this probably accounts for the ornithine content of the organism. Table 1 shows that 92·1 % of the ornithine estimated in the hydrolysed organism is found free in the disintegrate; allowing for the experimental errors involved in the various procedures it seems probable that all the hydrolysate ornithine arises from free ornithine carried down in the internal environment of the cells and that this amino-acid does not exist in a combined form in the protein of the organism.

By the use of this method for the estimation of the free amino-acid in the internal environment of the cells, it is possible to study the factors involved in the accumulation of such amino-acids within the internal environment and in their passage across the cell wall.

Diffusion of amino-acids out of the streptococcal cell

General method. Organisms grown in medium A, cells washed once, the internal amino-acid assayed, the cells then suspended in water or salt solution at a final cell suspension strength of *c.* 1 mg. dry weight cells/ml.; after a suitable interval the cells are centrifuged down and made up into a thick suspension for comparative assay.

When *Strep. faecalis* is grown in medium A the cells can be shown to possess free lysine and glutamic acid in their internal environment. To investigate whether this free amino-acid is able to diffuse out of the cells, a thick suspension of washed cells was taken, the internal lysine and glutamic acid assayed as described, and the cells then poured into distilled water at 0° to give a final cell suspension of approx. 1 mg. dry weight/ml. After 24 hr. the cells were centrifuged down and the internal amino-acid contents again determined. There appeared to be little or no loss of either lysine or glutamic acid from inside the cells. It was possible that the cells had been damaged by suspension in distilled water so the experiment was repeated, using the salt solution described as suspending medium. Samples of the cell suspension were centrifuged down at intervals and the internal lysine and glutamic acid estimated. Table 2 shows that over a period of 48 hr. at 2° there was no loss of internal lysine and about 25 % loss of internal glutamic acid. It would seem that there is no free diffusion of the amino-acids out of the cells. Table 2 also shows that the effect is not dependent on external pH within physiological limits although there is some loss of lysine when the external pH is 4·5.

Variation of internal amino-acid content with age of culture

The amount of free amino-acid existing in the internal environment of the cell at any moment must represent the balance between the amount passing through the cell wall from the external environment and the amount being metabolized either by breakdown or by synthesis into bacterial protein. To determine how the amount of free lysine or glutamic acid within the cell varied with the growth of the cell, samples of culture were taken during growth from a large-scale culture in medium A, the organisms centrifuged down, washed and the internal amino-acid content estimated. Table 3 shows the results expressed as μl. lysine or glutamic acid in the internal environment of 100 mg. dry weight of cells. Cells taken after the culture had been growing at 37° for 2½ hr. showed no lysine but a high concentration of internal glutamic acid. Cells harvested during the growth of the culture showed an increasing internal content of both lysine and glutamic acid, reaching a maximum value at the period of cessation of active cell division. After growth had ceased there was a small loss but this was not marked until after 24 hr.; this loss may be due to autolysis of a proportion of the cells. When it is desired to produce cells containing a high internal content of free amino-acid (Gale & Taylor, 1947) the cells should therefore be harvested soon after the cessation of growth in medium A.

Table 2. (*a*) *Loss of lysine and glutamic acid from the internal environment of* Strep. faecalis *cells on standing in amino-acid-free salt solution*

		μl. amino-acid in internal environment assayed on 95 mg. dry weight of cells	
Temp.	Time (hr.)	Lysine	Glutamic acid
2°	0	152	208
	3	158	224
	6	158	200
	24	152	176
	48	161	152
37°	0	152	208
	6	143	198

(*b*) *Effect of external* pH *on loss of amino-acids as above at* 2° *for* 24 *hr.*

Cells suspended in salt solution so that final concentration = approx. 1 mg./ml. during incubation.

	μl. amino-acid in internal environment assayed on 78·4 mg. dry weight of cells	
External pH	Lysine	Glutamic acid
Initial content	145	102
4·5	74	88
5·5	118	108
6·5	126	108
7·5	129	110
8·5	102	93
9·5	98	100

Table 3. *Variation of internal concentration of free amino-acids with age of culture*

			Internal concentration of *l*-(+)-lysine		Internal concentration of *l*-(+)-glutamic acid	
Age of culture (hr.)	Growth mg. cells/ml. medium	Cell sample (mg.)	Per sample (μl.)	Per 100 mg. cells (μl.)	Per sample (μl.)	Per 100 mg. cells (μl.)
2½	0·052	19·5	Nil	Nil	36	185
4	0·316	22·4	12	54	41	184
6	0·440	20·8	45	216	41	197
8	0·530	22·2	50	225	57	256
10	0·530	28·6	61	214	58	203
24	—	21·2	39	184	18	57

Passage of amino-acids into streptococcal cells

The cells used hitherto were grown in the amino-acid-rich medium A and were found to possess a high internal content of certain free amino-acids. When the cells were grown in an adequate medium in which the amino-acid content was just sufficient to supply growth needs, e.g. medium B, the internal content of lysine, glutamic acid and histidine was greatly decreased (Table 4).

Table 4. *Variation of internal concentration of free amino-acid with nature of growth medium*

	Growth medium A		Growth medium B	
Amino-acid	μl./ml. medium	μl. in internal environment of 100 mg. cells	μl./ml. medium	μl. in internal environment of 100 mg. cells
l-(+)-Lysine	150–200	170–200	2–3	15–25
l-(+)-Glutamic acid	150–180	200–300	2–5	9–15
l-(−)-Histidine	45–60	20–35	1–2	3–5

Such cells are deficient in internal amino-acids as compared with those harvested from medium A and will be referred to below as 'deficient cells'. By suspending such deficient cells, after washing, in solutions of various amino-acids it is possible to follow the appearance of the amino-acids in the internal environment and to study the factors involved in their passage across the cell wall. The results vary with the age of the culture and the best effects of the type described below are again obtained with cells harvested at about the end of active cell division. In medium B cells should be harvested after about 8–10 hr. growth at 37°.

General method. Organisms grown in medium B, cells washed once, the internal amino-acid assayed, the cells suspended in salt solution containing known concentrations of amino-acid with or without glucose (final suspension strength = approx. 1 mg./ml.), after suitable treatment the cells are centrifuged down and made up into thick suspension for comparative assay.

Lysine

When a thick washed suspension of *Strep. faecalis* cells grown in medium B is assayed for internal lysine, the cells suspended in a solution of lysine (100 μl./ml., as above) and then centrifuged out of solution at intervals for assay, it is found that the internal lysine content begins to increase immediately the deficient cells come into contact with the amino-acid. Fig. 2 shows that the internal lysine increases rapidly and reaches an equilibrium concentration within 20–30 min. of contact at 10° and pH 7·2. This rapid rate of passage of lysine into the cell is in marked contrast to the complete absence of outward diffusion previously studied. The position of the experimental points on the time scale is difficult to determine with accuracy as the organisms have to be centrifuged out of the lysine solution before assay is possible. The cells are usually centrifuged at *c.* 1500 × g. for 6 min. in the cold and then washed in ice-cold water before assay so that the abscissae which represent the time for which the cells were held in lysine solution prior to centrifuging should probably be increased by 6 min.

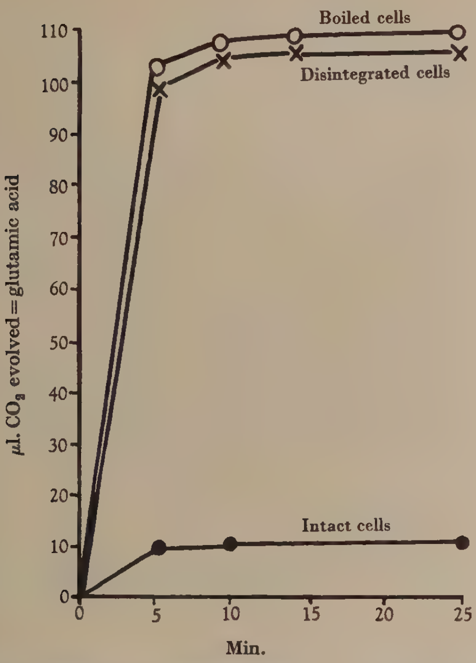

Fig. 1. Action of glutamic acid decarboxylase on intact and disrupted cells (*Strep. faecalis* grown in medium A). Each estimation carried out on 60 mg. dry weight of cells.

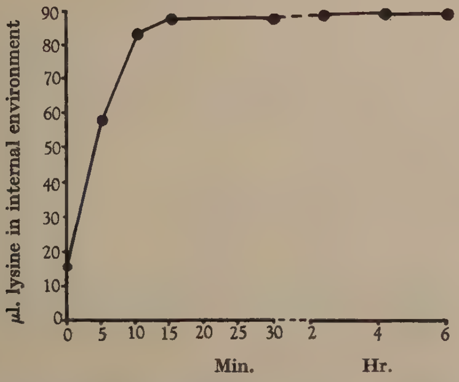

Fig. 2. Rate of appearance of lysine in internal environment of deficient *Strep. faecalis* cells standing in lysine solution (300 μl./ml.) at 10°. External pH = 7·2. Internal lysine assayed on 82·0 mg. dry weight of cells.

The equilibrium concentration of lysine reached in the cell is dependent upon the lysine concentration in the external environment. This can be shown by suspending deficient cells in solutions of lysine of different concentrations and centrifuging the cells down after allowing a sufficient time (3 hr.) for equilibrium to be reached in all cases. Fig. 3 shows the variation of the internal concentration at equilibrium with increasing external lysine concentrations.

Effect of external lysine concentration on rate of entry of lysine. Fig. 2 shows that lysine enters the cell rapidly during the first 20 min. of contact. If samples are taken after 5 or 10 min. of contact it is possible to study the influence of various environmental factors on the rate at which lysine is entering the cell. Fig. 4 shows the effect of increasing lysine concentration on the rate of entry into the deficient cell. Within the concentration range studied the rate of entry increases with increasing external concentration.

Effect of pH *on the rate of entry of lysine.* At pH 4·5 lysine enters the cell slowly and the rate increases with increasing pH up to 9·5 which represents the limit of alkali tolerance for this organism (Fig. 5). The isoelectric point for lysine is 9·47 and the results shown in Fig. 5 suggest that lysine passes most easily into the cell in the isoelectric form. The final equilibrium concentration reached inside the cell after 24 hr. at 2° is independent of the external pH within experimental error.

Effect of temperature on rate of lysine entry. Lysine enters the cells at an appreciable rate at 0° and the rate increases with temperature (Fig. 6), the temperature coefficient over the range 20–30° being 1·40, which is only slightly greater than that which would be expected for the free diffusion of lysine (Davson & Danielli, 1943). It is possible that the pH effects described are due to an alteration in the permeability of the cell wall with pH, in which case the temperature coefficient should also alter with pH. The value was determined with cells suspended at an external pH of 5·5 and the value obtained, 1·35, is not significantly different from that determined at pH 7·4.

Concentration of lysine within the cell. A knowledge of the volume available for amino-acids in solution in the internal environment of the cell (the internal 'free-space') is necessary for calculation of the actual concentration of lysine inside the cell. An attempt to determine this volume has been made as follows.

The wet volume was measured directly by preparing a thick suspension of washed streptococcal cells and placing measured amounts into accurately graduated centrifuge tubes made from sealed pipettes. These were then embedded in rubber and centrifuged on an Ecco-Blitz supercentrifuge at 7000 g. until there was no further diminution in the volume of the solid material. After 1½ hr. the meniscus had ceased to move in the tubes and the volume of the intact cells was read off. The dry weight of the original suspension was determined as usual so that it was then possible to calculate the volume occupied by a known weight of intact cells.

It is reasonable to assume that the cells are composed largely of protein and since the specific volume of proteins lies between 0·70 and 0·75 it is possible to calculate the approximate volume occupied by the solid material of the cells. In order not to minimize the volume of the internal free space, the value of 0·70 was taken for the specific volume of the solid material. A series of experimental determinations give the volume occupied by 100 mg. intact cells = 0·347 ml., volume of solid material = 0·07 ml., consequently volume of internal

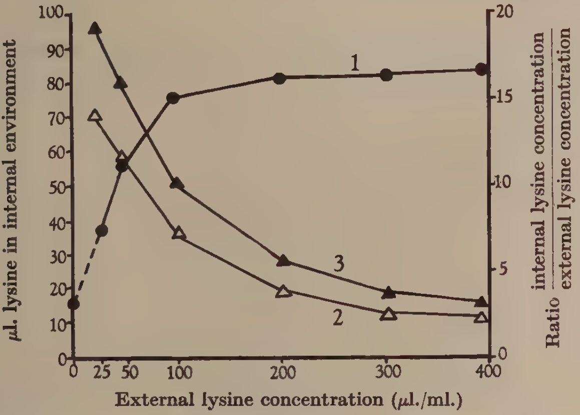

Fig. 3. Effect of external lysine concentration on (1) internal lysine concentration ●—●, (2) and (3) ratio of internal/external lysine concentrations. (2) △—△, calculation based on volume of intact cell, (3) ▲—▲, calculation based on volume of (intact cell less solid debris). Cells suspended in lysine solutions as shown for 3 hr. at 4°. Lysine assayed on 28·0 mg. dry weight of cells in each case.

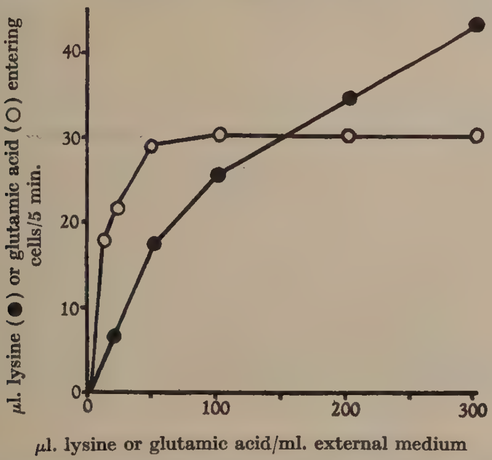

Fig. 4. Effect of external concentration of lysine or glutamic acid on rate of appearance of these amino-acids in the internal environment of deficient cells. ●—●, lysine: temperature = 10°; lysine assayed on 30·8 mg. dry weight cells. ○—○, glutamic acid: temperature = 37° in pressure 0·5% glucose; glutamic acid assayed on 27·2 mg. dry weight of cells. Initial pH = 7·2 in all cases.

'free-space' = 0·277 ml. On the basis of these calculations, the internal lysine concentrations are represented by the values given in Fig. 3. Curves 2 and 3 show the variation with the external lysine concentration of the ratio of the internal to the external concentration. In curve 2 values are calculated on a basis of the total volume of the intact cell (0·347 ml./100 mg.); in curve 3 on a basis of the volume of the free space (0·277 ml./100 mg.). The actual value in each case will presumably lie between the two curves. It can be seen that the internal concentration is three to four times greater than the external when the latter is of the value of 300 μl./ml. and the ratio rises to fifteen to twenty times when the external concentration is 25 μl./ml. Thus although the absolute concentration within the cell is lower for small external concentrations than for high, there is an actual concentrating action across the cell wall which increases as the external lysine decreases. The actual concentration of the internal lysine when that in the external environment is equal to 300 μl./ml. is 0·035–0·05 M.

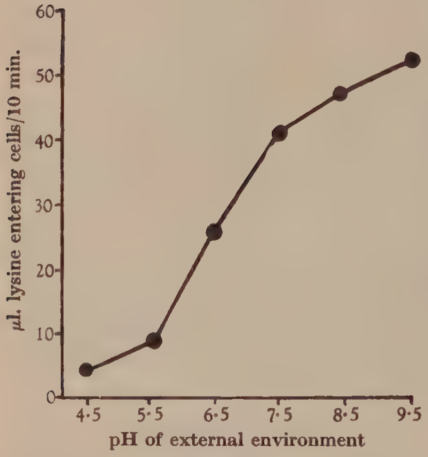

Fig. 5

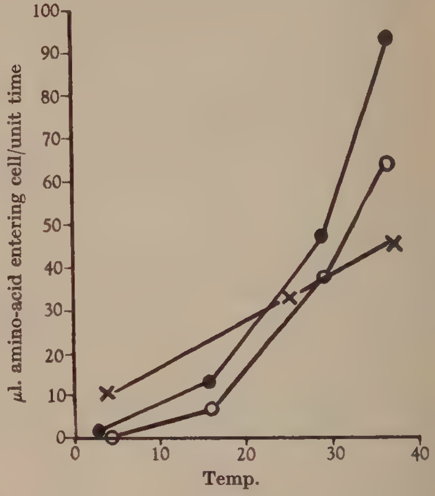

Fig. 6

Fig. 5. Effect of pH on the rate of appearance of lysine in the internal environment of deficient cells standing in lysine, temperature = 10°. External lysine concentration = 100 μl./ml. Lysine assayed on 21·2 mg. dry weight of cells in each case.

Fig. 6. Effect of temperature on rate of entry of: ×—×, lysine—amount entering 33·2 mg. cells in 5 min.; ●—●, glutamine—amount entering 25·2 mg. cells in 15 min.; ○—○, glutamic acid—amount entering 25·2 mg. cells in 15 min. External concentration = 100 μl. amino-acid/ml.

Glutamic acid and glutamine

The results obtained in the experiment illustrated in Fig. 4 suggest that lysine passes into the cell most easily at its isoelectric point. The passage of glutamic acid into the cell was next studied as the isoelectric point of this amino-acid lies at 3·22.

Suspensions of deficient cells were therefore diluted with solutions of glutamic acid the pH values of which were adjusted to cover the range 4·0–9·5. The suspensions were then incubated for 3 hr. at 37° and also at 2°, the organisms then centrifuged down and the internal glutamic acid content assayed.

In no case was there any significant increase in the internal glutamic acid content. McIlwain, Fildes, Gladstone & Knight (1939) showed that certain haemolytic streptococci were unable to grow in the absence of glutamine although glutamic acid is present. It is possible that glutamine (isoelectric point 5·56) passes the bacterial cell wall more easily than glutamic acid so the experiment was repeated with glutamine; the use of the decarboxylase preparation on boiled cells does not distinguish between glutamine and glutamic acid. Although there was a small increase in the glutamine content of the cells under the experimental conditions used, this was scarcely significant. When, however, deficient cells were placed in casein digest, with or without glucose, there was a marked increase in the internal glutamic acid concentration, this increase being greater in the presence of glucose than in its absence. Finally, it was found that when deficient cells were incubated in glutamic acid solution containing glucose there was a rapid increase of internal glutamic acid. There was, however, no increase if the incubation was carried out at 0°; this fact enables the rate of entry of glutamic acid into the deficient cell to be studied with greater accuracy than was possible with the lysine system, as the reaction can be checked in this case by lowering the temperature to 0°.

The rate of glutamic acid entry. A series of centrifuge tubes were put up each containing 25 ml. of glutamic acid (200 μl./ml.) in salt solution and incubated at 37°. When a steady temperature had been attained, 2·5 ml. of thick suspension of deficient cells (20–30 mg. dry weight of cells per ml.) were pipetted into each tube. At the end of 5, 10, 20 and 30 min. approx. 20 g. of frozen distilled water was dropped into one tube and the organisms immediately centrifuged down and washed once in ice-cold water before assay.

The results are shown in Fig. 7. There was no increase in internal glutamic acid or glutamine in the absence of glucose. In the presence of glutamine and glucose in the external environment, glutamine (or glutamic acid—the decarboxylase preparation does not distinguish) entered the cell from the time of contact and the internal concentration reached an equilibrium value within 20–30 min. In the presence of glutamic acid and glucose in the external environment, glutamic acid entered the cell but there appeared to be a short lag phase before glutamic acid entered and the final equilibrium was reached in 30 min. after contact. Using equivalent external concentrations of glutamic acid and glutamine, the final internal concentration reached was the same in both cases. The lag period could not be demonstrated with cells harvested earlier in the growth period.

In the preliminary tests it was shown that the internal glutamic acid content of deficient cells was raised by incubation in casein digest whether or not glucose was present. This suggested that glucose acts as a source of energy necessary for the transfer of glutamic acid across the cell wall and that glucose was not the only available energy source, some other substance in casein digest being able to act as such. Casein digest consists mainly of amino-acids and the amino-acid catabolism of these streptococci is limited to the breakdown of arginine by arginine dihydrolase (Hills, 1940) and of tyrosine by tyrosine decarboxylase (Gale, 1940). The former is an exothermic reaction and it is possible that arginine may therefore act as energy source for the transfer of glutamic acid into the cell. Fig. 7 shows the increase in internal glutamic acid that resulted from suspension of the deficient cells in glutamic acid +0·1 % arginine. It can be seen that glutamic acid entered the cell under these conditions although the rate of entry was considerably less than that achieved when glucose was present as energy source.

Concentration of glutamic acid within the cell. When deficient cells were incubated at 37° in glutamic acid-glucose-salt solutions, the final concentration of glutamic acid attained inside the cell was dependent upon the external glutamic acid concentration (Fig. 8). Knowing the volume of the internal environment (determined as described above) the ratio of the internal to external concentration can be calculated. The curves show that this ratio rises from 3–4 for external values of 300 μl. glutamic acid/ml. to 50–60 for external values of 10 μl./ml. The internal concentration when the external value =300 μl./ml. is approx. 0·04–0·06 M. It appears that, again, a concentration mechanism is brought into play as glutamic acid passes across the cell wall into the cell which is highly effective when the external concentration is low.

Table 5. *Gradient in glutamic acid concentration across the cell wall*

Internal glutamic acid concentrations calculated on the basis that 100 mg. intact cells occupy a volume of 0·347 ml.

External concentration of glutamic acid (μl./ml.)	Internal concentration of glutamic acid (μl./ml.)	Gradient across cell wall (μl.)
10	506	496
25	556	531
50	730	680
100	836	736
200	927	727
300	1058	758
400	1190	790

Further light on this concentration mechanism is obtained when the data are presented in a different form. When the internal concentration of glutamic acid and the difference between the internal and external concentrations are calculated the values shown in Table 5 are obtained. It can be seen that the gradient from external to internal concentrations across the cell wall is approximately constant for external values greater than 100 μl./ml. Below this there is a fall in the gradient although this may be less than that recorded, as experimental error increases with decreasing external concentration. It is possible that the cell wall maintains a constant gradient between internal and external concentrations and that this gradient is maintained by some sort of secretory mechanism. If the same treatment is applied to the values obtained for lysine (Fig. 3), where the passage of the amino-acid across the cell wall appears to be one of diffusion, values for the gradient across the cell wall are obtained which are not even approximately constant.

Effect of pH *on the rate of entry of glutamic acid into the cell.* By taking samples of deficient cells within 10 min. of contact with standard glutamic acid glucose salt solutions, it is possible to study the effect of variation of the

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external environment on the rate of entry of glutamic acid. All these experiments were carried out in duplicate, one set with glutamic acid in the external environment, and the other with an equimolar concentration of glutamine. In no case was there any significant difference in the results obtained; the glutamic acid values are quoted in the figures. Fig. 9 shows the effect of the external pH on rate of entry of glutamic acid. The various solutions were buffered with phosphate buffers and pH determinations made at the beginning and end of

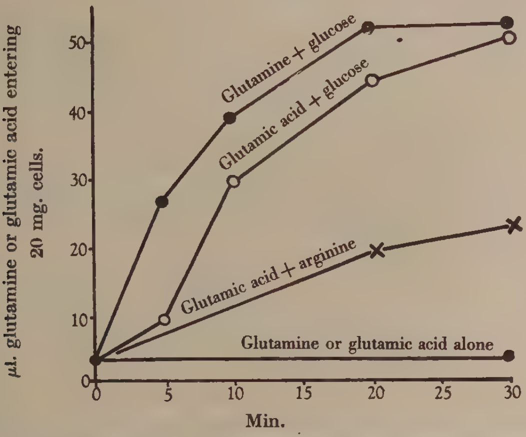

Fig. 7. Rate of entry of glutamine or glutamic acid into deficient cells in presence and absence of glucose or arginine. Temperature = 37°. External concentration of glutamine/glutamic acid = 200 μl./ml. Initial pH = 7·2. Dry weight of cells assayed = 20·0 mg.

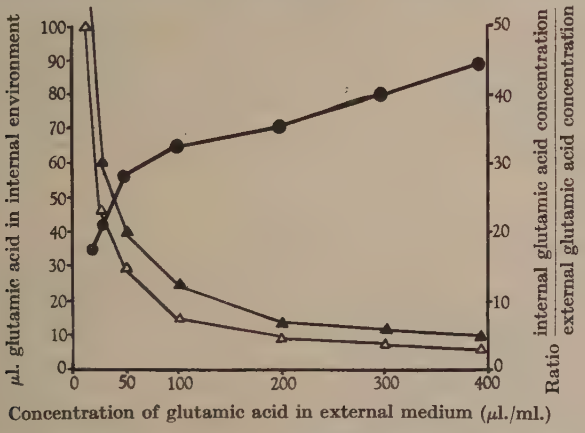

Fig. 8. Effect of external glutamic acid concentration on (*a*) internal glutamic acid concentration ●—●, (*b*) ratio of internal/external glutamic acid concentrations. △—△, calculation based on volume of intact cell; ▲—▲, calculation based on volume of (intact cell − solid debris). Cells suspended in glutamic acid solutions (containing salts and 0·5% glucose) as shown for 3 hr. at 37°. Initial pH = 7·4. Glutamic acid assayed on 22·8 mg. dry weight of cells (14 hr. culture).

each experiment. When glucose was present as energy source, the rate of glutamic acid entry was small in the acid range, rose to an optimum at *c.* pH 6·5 and fell less steeply in the alkaline range, the rate still being rapid at pH 8·5. The shape of the curve was essentially a reflexion of the effect of pH on the glucose fermentation system and did not give any information about the variation of cell wall permeability with pH towards glutamic acid. With arginine as energy source again the results seemed to depend largely upon the sensitivity of the energy-providing system to pH variation (Fig. 9, lower curve), although there was a suggestion that the complete system was more effective as the limit of alkali tolerance was approached. In general, the use of arginine as energy source was unsatisfactory and gave variable results; in later studies glucose was used as energy source and the external environment initially adjusted to *c.* pH 7·5.

Effect of temperature on rate of entry of glutamic acid. At 2° there was no significant increase in the internal glutamic acid concentration over a period of 3 hr. The rate increased rapidly with a rise of temperature, the temperature coefficient over the range 20–30° being 1·94. This temperature curve (Fig. 6) is typical of an enzyme system (presumably the controlling factor being the rate of glucolysis) and is markedly different from that given by the lysine entry curve which represents the effect of temperature on simple diffusion. Glutamine gave a similar temperature curve (temp. coefficient 2·35 though the experimental error was so great that this difference may not be significant).

Effect of external glutamic concentration on rate of entry. The curve for glutamic acid (Fig. 6) is markedly different from that given for lysine in that the rate rapidly reached a maximum value above which increasing external concentrations had no effect on the rate of entry, suggesting the relation existing between the rate of an enzyme action and its substrate concentration rather than that of diffusion across a membrane as shown by lysine.

Histidine

Table 6 shows that a Group D *Streptococcus* grown in medium A had free histidine within the internal environment although the concentration was less than that of lysine or glutamic acid. Cells grown in medium B contained little or no free histidine. Experiments similar to those described were therefore made on the histidine content of the internal environment of deficient cells incubated in histidine solutions. Table 6 shows that there was no increase in the internal environment when the cells are incubated at 0° or 37° in histidine-salt solutions but a small increase when glucose was also present in the suspension phase. Histidine therefore resembles glutamic acid in that it cannot pass into the cell in the absence of a source of energy. Table 6 also shows the effect of external histidine concentration on the internal concentration, and the ratio of the internal to external concentration calculated as previously described. It can be seen that this ratio varied between 2 and 0·5 with external concentration, suggesting that, if allowance be made for experimental error, the internal concentration was approximately equal to that in the external environment, so that there was no concentration effect across the cell membrane as there was in the case of glutamic acid. The magnitude of the effect is so small in this case that no further attempts were made to study the factors involved.

Aspartic acid

No aspartic decarboxylase has yet been discovered so that it has not been possible to investigate whether the passage of aspartic acid across the cell wall occurs under conditions similar to those required by glutamic acid. An attempt was made to determine whether the process took place in the presence or absence of glucose by the use of chloramine T as a means of estimating aspartic acid.

Table 6. (*a*) *Uptake of* l-(−)-*histidine in the internal environment of deficient cells*

Dry weight of cells assayed = 33·4 mg. Cells suspended for 3½ hr. in salt solution containing 300 μl. *l*-(−)-histidine/ml., initial pH = 7·2, other conditions as below.

Treatment	Histidine in internal environment (*a*)	(*b*)
Initial	0	2
Incubation at 4°	2	—
Incubation at 37°	4	6
Incubation at 37° in presence of 0·5 % glucose	7	13
Incubation at 37° in presence of 0·2 % arginine	—	5

(*b*) *Effect of external histidine concentration on concentration within internal environment. Concentrations calculated on a basis of* (1) *volume of intact cell;* (2) *volume of* (*intact cell − solid debris*) (see text)

Dry weight of cells assayed 39·2 mg. Conditions of incubation: cells suspended in salt solution (about 1·5 mg. cells/ml.) containing 0·5 % glucose and histidine as below for 3½ hr. at 37°.

External histidine concentration μl./ml.	Internal histidine concentration μl./ml. Basis 1	Basis 2	Ratio internal/external histidine concentrations Basis 1	Basis 2
300	119	169	0·4	0·56
200	112	158	0·56	0·8
100	90	127	0·9	1·27
50	82	116	1·64	2·3
25	52	74	2·08	2·98

Dakin (1917) showed that chloramine T reacts with amino-acids in such a way as to liberate CO_2 from the carboxyl group. In the case of aspartic acid, both carboxyl groups are thus attacked and Cohen (1940) used this fact to estimate aspartic acid manometrically although the method is non-specific. Deficient cells were therefore suspended in salt solutions containing aspartic acid with and without glucose under the usual conditions, incubated for 1 hr. at 37°, the organism centrifuged down, washed and taken up in 10 % H_2SO_4. After standing 0·5 ml. 10 % sodium tungstate solution was added and the protein precipitate centrifuged down. The supernatant liquid was collected in each case, neutralized to pH 4·5 and 'aspartic acid' estimated according to the method of Cohen (1940).

There was no increase in attacked —COOH groups in the organism incubated with aspartic acid alone but cells incubated in the presence of aspartic acid and glucose showed approx. 350 % increase in —COOH which probably represented aspartic acid. It is not possible to differentiate between internal and external aspartic acid by this method but the results suggest that aspartic acid resembles glutamic acid in that it passes into the cell only in the presence of a source of energy such as glucose.

Effect of ions in suspension medium

The experiments described above were carried out in the presence of a salt solution containing Na^+, K^+, Mg^{++}, PO''_4 Cl', SO''_4 and traces of Ca^{++}. Diffusion experiments conducted with other cells are often markedly affected by the presence of certain ions in the medium; consequently experiments were carried out to determine whether the uptake of lysine or glutamic acid was dependent upon the presence of any of the ionic constituents used. The effect of Ca^{++} was tested by investigating the uptake of glutamic acid in a medium carefully freed from traces of Ca^{++}, and in the same medium saturated with Ca^{++} to the point of the precipitation of calcium phosphate. To test the effect of omission of other ions parallel experiments were carried out in the usual salt mixture and in a medium containing 0·9 % NaCl and buffered by M/80-$NaHCO_3$ in the presence of 5 % CO_2 + 95 % N_2 gas mixture to give pH 7·2 under the experimental conditions. The presence or absence of Ca, Mg or SO''_4 had no significant effect on the uptake of glutamic acid by deficient cells; analysis of the simplified medium showed that it contained 4·6 μg. phosphorus/ml.

The uptake of lysine was independent of the presence of Mg^{++} or SO''_4 (Ca^{++} was not tested) and was unaffected by 0·01 M-HCN or iodoacetate, while the uptake of glutamic acid was inhibited by any substance interfering with glucolysis.

Passage of amino-acids out of the cells and attainment of equilibrium between internal and external environments

General method. The organisms were grown in medium A, the cells washed once and the internal amino-acid assayed, the cells suspended in salt solution + 1 % glucose to a final suspension strength of *c.* 1 mg./ml., after suitable treatment the cells were centrifuged down and made up into thick suspension for comparative assay.

Glutamic acid

The work previously described showed that glutamic acid passed from the external environment into deficient streptococcal cells until the internal concentration was considerably greater than that outside the cell, and that this internal amino-acid was not then free to diffuse out through the cell wall when the cells were incubated in distilled water. When, however, cells grown in medium A and containing a high internal concentration of glutamic acid were incubated in a salt solution + glucose, the internal concentration decreased (Fig. 10). The glutamic content of the cells dropped rapidly at first and then the rate decreased until, in the case quoted, the internal concentration had fallen to 34 μl./47 mg. cells assayed from an initial content of 105 μl./47 mg. cells.

No further decrease in internal content could be shown over a period of 4 hr. at 37°. In the absence of glucose in the external medium, no significant decrease in the glutamic content of the cells occurred.

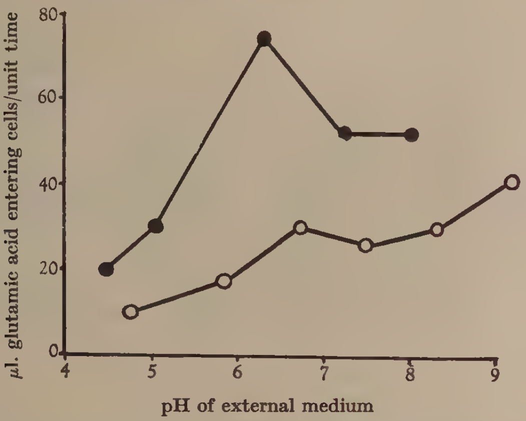

Fig. 9. Effect of external pH on rate of entry of glutamic acid. ●—● in 10 min. in presence of 0·5% glucose, ○—○ in 30 min. in presence of 0·1% arginine. External glutamic acid concentration = 100 µl./ml. Temperature = 37°. Dry weight of cells assayed = 25·0 mg.

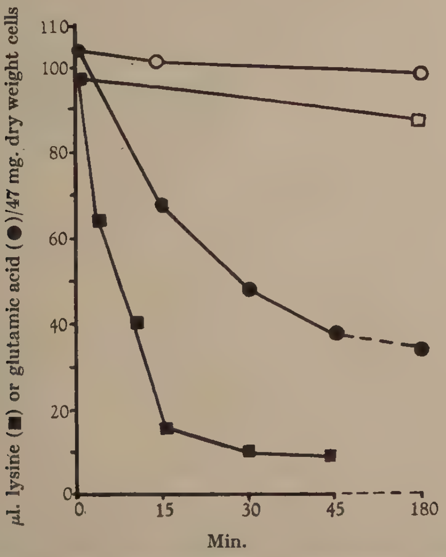

Fig. 10. Loss of lysine (■) and glutamic acid (●) from cells incubated at 37° in salt solution alone (□ ○) or with 1% glucose (■ ●). Assays on 47 mg. dry weight of cells.

The shape of the curve in Fig. 10 suggests that the glutamic acid concentration was approaching an equilibrium, probably by the passage of glutamic acid back through the cell wall until equilibrium was restored between internal and external environments. To determine whether this is the case or whether the glutamic acid disappearing is being metabolized, the experiment was carried out on a larger scale, the organisms centrifuged down after 3 hr., the supernatant solution concentrated *in vacuo* and assayed for glutamic acid. Table 7 shows that when the cells were suspended in salt solution alone under these

Table 7. *Passage of internal glutamic acid out of cell*

The cells were grown in medium A and made up into washed suspension of dry weight 40·5 mg./ml. One ml. was used for assay of initial glutamic content. The suspension was then diluted in salt solution as below to a final suspension strength of 4·05 mg./ml. and incubated for 3 hr. at 37°. The cells were then centrifuged out of suspension, washed once and made up into thick suspension of 40·5 mg./ml. for glutamic assay. The supernatant (external medium during incubation) was evaporated *in vacuo* and assayed for glutamic acid.

				Glutamic acid	
Treatment	Internal glutamic acid (μl./40·5 mg.)	Vol. of suspension diluted (ml.)	Glutamic acid in external medium (μl.)	Lost from organism (μl.)	Gain in supernatant (μl.)
Initial	87	—	0	—	—
Incubated in salt alone	79	3	0	24	—
Incubated in salt + 1 % glucose (1)	20	5	189	335	189
Incubated in salt + 1 % glucose (2)	24	5	139	315	139

Table 8. *Equilibrium between internal and external concentrations of glutamic acid*

Type of experiment	External concentration of glutamic acid μl./ml. (*a*)	Internal concentration of glutamic acid μl./100 mg. cells (*b*)	Ratio *b*/*a* (*c*)
Series A. Deficient cells incubated in glutamic acid + glucose solutions until equilibrium is reached at 37°	10	134	13·4
	25	162	6·5
	50	239	4·8
	100	274	2·7
	200	304	1·5
Series B. Cells grown in full medium (A) and assayed while in equilibrium with medium at harvesting	121	211	1·7
	165	230	1·4
Series C. Cells grown in full medium (A) and then incubated in glucose or in glucose + glutamic acid solutions until internal value drops to equilibrium at 37°	3	60	20·0
	4·5	50	11·2
	5·5	61	11·0
	6·5	81	12·5
	21	83	4·0
	130	113	0·9

conditions, no glutamic acid was found in the supernatant but when glucose was present during the incubation, glutamic acid passed out of the cells and could be found in the supernatant. The amount of glutamic acid in the supernatant accounted for 50–60 % of that disappearing from the internal environment during the incubation; it seems probable that the remainder was metabolized (this will be dealt with in a later paper). The presence of a small amount of glutamic acid in the suspending medium resulted in a greater retention of glutamic acid in the internal environment. Table 8 shows the amount of glutamic acid held in the internal environment of 100 mg. cells when in equilibrium with three different external environments. In the cases quoted in series C the values of the external concentration of glutamic acid were obtained by direct assay of the external environment after incubation. The ratio of the internal concentration to external concentration varied as described in the previous section. For external concentrations of approx. 10 μl. glutamic acid/ml. the empirical ratio calculated in column (*c*) is approx. 13 for experiments carried out as in series A; for the reverse type of experiment as in series C the ratio is approx. 12. The ratio fell with rising external concentration, being approx. 6 for 25 μl. external (series A) and approx. 4 for 21 μl. external (series C). The agreement between these values, whether approached as in series A or C, gives convincing evidence that for any given external glutamic acid concentration there is an equilibrium internal concentration and that the internal concentration will adjust to this value whether it is initially higher or lower and as long as glucose (or some other source of energy) is available.

The ratio values given in series B are in good agreement with those obtained in series A for similar concentrations, showing that the equilibrium conditions are the same whether the external environment is a complicated one as in the growth medium or a simple one as in the experiments of series A.

Early in these investigations it was found that satisfactory results, either with regard to a high internal amino-acid content of cells grown in medium A (Table 3) or for satisfactory amounts of lysine or glutamic acid taken up by deficient cells, were only obtained with cells harvested early in the growth period. This suggested that the equilibrium between internal and external environments altered as the cultures aged.

A large-scale culture was grown in medium B and samples taken at intervals throughout the growth period. The cells were centrifuged out of each sample, washed once and then incubated for 40 min. at a suspension strength of *c.* 1 mg./ml. in salt solution containing 200 μl. glutamic acid/ml. and 0·5 % glucose. After this treatment, the cells were centrifuged down, washed once and the internal glutamic acid content assayed as usual.

Table 9 shows that the amount of glutamic acid taken up per 100 mg. cells under these conditions varied greatly with the age of culture, being very high with cultures taken as soon as possible after inoculation and then decreasing steadily throughout the growth period and after. Since the external concentration of glutamic acid was the same in all these experiments, it follows that the efficiency of the concentrating effect across the cell membrane is greatest in rapidly growing cells and decreases when growth ceases. This must also mean that the internal concentration of glutamic acid in equilibrium with a given external concentration is very high in cells early in the growth phase.

Lysine

As shown previously there was no diffusion of lysine out of streptococcal cells grown in medium A. Since glutamic acid passed out of the cell during glycolysis it was of interest to determine whether lysine would also pass out of the cell under these conditions. The experiments described in the previous section were therefore repeated and the internal lysine content of the cells followed. Incubation of cells harvested from medium A for 3 hr. in salt solution at 37° resulted in no significant loss of lysine from the internal environment but when glucose was added to the external medium, there was a marked disappearance of lysine within the cells. The internal lysine did not fall to negligible proportions and about 60 % of the lysine disappearing from the internal environment could be recovered from the external medium at the end of the experiment. The loss of internal lysine from the cells in the presence of glucose was (Fig. 10) considerably more rapid than the corresponding loss of glutamic acid. The shape of the curve suggests that the final condition represented some state of equilibrium.

Table 9. *Effect of the age of culture on the amount of internal glutamic acid in equilibrium with a given external concentration*

Organisms grown in medium B and sampled at intervals. Temperature = 37°. Organisms centrifuged out of culture, washed once, and then incubated at 37° for 40 min. in salt solution + 0·5 % glucose + glutamic acid (200 μl./ml.). Cells then centrifuged down, washed and assayed for internal glutamic acid.

			μl. glutamic acid at equilibrium	
Age of culture (hr.)	Growth (mg./ml. medium)	Dry wt. of cells assayed (mg.)	Per sample (μl.)	Per 100 mg. cells (μl.)
2·5	0·050	6·5	39	600
3	0·15	12·4	58	460
4	0·222	21·0	80	380
6	0·300	31·4	76	241
8	0·306	26·4	66	260
10	0·306	25·4	81	320
11	0·306	25·0	68	212
12	0·306	30·0	32	107
14	0·306	23·2	28	121
18	0·300	19·6	36	132
24	—	31·6	34	108

Relation between glutamic acid and lysine uptake by deficient cells

The facts discovered so far can be summarized as follows:

1. Glutamic acid cannot diffuse across the cell wall in either direction except in the presence of a source of energy (e.g. glucose); glutamic acid then passes across the cell wall in either direction until an equilibrium is set up between the internal and external concentrations, the former being greater than the latter.

2. Lysine will diffuse into the deficient cell but is unable to leave the 'saturated' cell suspended in lysine-free salt solution.

3. Lysine will pass out of the 'saturated' cell into an environment deficient in lysine but only when energy is supplied to the system from glycolysis, etc.

The passage of lysine across the cell wall therefore appears at first sight to involve some unsymmetrical arrangement which allows diffusion across the cell wall in an inward direction only. The question therefore arises whether the inward diffusion of the lysine is, despite its kinetics, due to some residual metabolism. To test this the effect of the presence of glucose on (*a*) the rate of inward passage of lysine, and (*b*) the final equilibrium conditions existing between the internal and external lysine concentrations was examined. The first point was difficult to investigate because the rate of lysine entry was so rapid at temperatures at which glycolysis would take place at a significant rate; it was not possible to determine any effect of glucose on the rate of lysine entry into deficient cells at 25°. The presence of glucose greatly decreased the amount of lysine that the cell could take up (Table 10).

Table 10. *Effect of glucose on internal concentration of lysine attained in deficient cells suspended in lysine solutions*

Cells grown in medium B and lysine assayed on samples of 32·5 mg. dry weight. Cells suspended in salt solutions containing lysine and glucose as below, final suspension strength = 3·25 mg./ml. Incubation period = 3 hr. at 25°

External concentration of lysine (μl./ml.)	Internal lysine concentration at equilibrium (μl./32·5 mg. cells)	
	(*a*) Glucose absent	(*b*) Glucose present (0·5 %)
25	23	3
50	38	4
100	42	4
400	45	3

Table 11. *Effect of glucose and glutamic acid on the uptake of lysine by deficient cells*

Cells grown in medium B and made up into washed suspension of strength 33·0 mg. dry weight of cells/ml. Cells diluted in salt solution with additions as below to final suspension strength = 3·3 mg./ml. and incubated for 1 hr. at 37° (first treatment) followed by 2 hr. at 25° (second treatment). Cells centrifuged out of suspension and lysine assayed on 33·0 mg. Concentrations: lysine 200 μl./ml.; glutamic acid 200 μl./ml.; glucose 0·5 %; salt mixture as usual.

	First treatment	Second treatment	Internal lysine μl./33·0 mg. cells
1.	—	Lysine alone	50
2.	—	Lysine + glucose	12
3.	—	Lysine, glucose, glutamic acid	35
4.	Glucose	Lysine alone	14
5.	Glutamic acid	Lysine alone	42
6.	Glucose + glutamic acid	Lysine alone	33
7.	Glucose + glutamic acid	Lysine + glucose	14

These results were obtained with a simple system consisting of salts, lysine and glucose and do not accord with those obtained in the more complex system of the full-growth medium since cells harvested from this medium contained approximately equimolar amounts of both lysine and glutamic acid although growth had taken place in the presence of excess glucose. The presence of glucose decreased the uptake of lysine by the cells but the presence of both glucose and glutamic acid restored, although not completely, the lysine uptake (Table 11). The system was further analysed by treatment of the cells with glutamic acid and/or glucose prior to incubation with lysine and Table 11 shows that treatment with glucose alone decreased the internal lysine uptake, glutamic acid alone had no effect, while pretreatment with glucose and glutamic acid partially abolished the depressant action of glucose alone.

These results suggest that whatever the system is that is responsible for the uptake of lysine by the deficient cells it was inactivated or abolished by the presence of glucose but protected from this inactivation by a simultaneous or previous assimilation of glutamic acid.

DISCUSSION

It has been shown that: (*a*) the streptococcal cells used had an internal environment in which certain amino-acids existed in a 'free' state; (*b*) some mechanism exists whereby lysine and glutamic acid may exist in a more concentrated solution inside the cell than outside under equilibrium conditions; (*c*) the passage of lysine into the cell across the cell wall appears to be one of simple diffusion; (*d*) the passage of glutamic acid, glutamine, aspartic acid and, probably, histidine into the cell requires energy which can be supplied by some exergonic reaction such as fermentation or the breakdown of arginine.

The conditions under which a substance can exist in a more concentrated solution on one side of a membrane than on the other are not clearly understood. Where the substance can pass across the membrane by diffusion as appears to be the case with lysine three conditions may hold: (1) where there are several forms in equilibrium on either side of the membrane then the concentration of the form which actually passes through the membrane must be the same on both sides of the membrane, (2) the substance must enter into combination with some other component of the system on one side of the membrane or (3) the substance must be held in some electrostatic combination on one side of the membrane. Until more is known about the conditions of ionization across the membrane, it is not possible to give a decision on the first possibility. Since, however, the equilibrium concentration of lysine reached within the cell when the external concentration was constant was independent of the external pH, it seems improbable that this could be the explanation. There remains the possibility that the internal lysine is combined chemically with some internal component of the cell. Since the amino-acid decarboxylases cannot react with their amino-acid substrates unless the three polar groups of the substrate molecule are in a free unsubstituted condition (Gale, 1946*b*) any chemical combination must be of a very labile nature or the amino-acids would not be estimated by the method used.

There remains the possibility that the lysine is held in the cell by electrostatic forces. Bacteria in general are negatively charged (Winslow, Falk & Caulfield, 1924) and the lysine molecule carries a positive charge under the conditions of these experiments. It seems probable that the diffusion of lysine into the cell is due to electrostatic attraction. The effect of glucose in decreasing the lysine uptake could thus be explained as a decrease in electro-negative charge in the cell as a result of glucolysis. It is well known that glucolysis is accompanied by the migration of various ions across the cell wall. Thus Leibowitz & Kupermintz (1942) showed that K ions enter *Bact. coli* cells when glucolysis begins. Conway (1942) explains this as follows: the cell membrane is permeable to K or smaller ions but impermeable to Na or larger ions, permeable to phosphate but not to phosphate esters of glucose, etc. When glucose enters the cell, it is esterified and the effective product of K and phosphate ions falls so that both ions then enter the cell. The esterification of phosphate within the cell may lead to a decrease in the negative charge and consequent decreased attraction for lysine ions. This attraction would be increased again if there were an inward migration of negative ions at the same time as glucolysis occurs; this happens when glutamic acid is present in the external medium. The explanation of the decreased uptake of lysine in the presence of glucolysis and the partial abolition of this decrease by the further addition of glutamic acid probably lies in ionic transfers of this nature. If the lysine passes into the cell as a result of electrostatic attraction, it follows that it will not diffuse out unless modification of the internal charge occurs. When cells containing high concentrations of both lysine and glutamic acid are incubated in an amino-acid-free medium containing glucose, then a further series of migrations takes place in the course of which both lysine and glutamic acid pass out of the cell. The attainment of glutamic acid equilibrium across the cell wall will alter the charge in the cell and the capacity to fix lysine, although comparison of the rates at which lysine and glutamic acid pass out of the cell (Fig. 10) would appear to indicate that the loss of glutamic acid itself is not the controlling factor in lysine loss or retention in this case.

It is clear that neither glutamic acid nor glutamine can pass across the cell wall unless energy is supplied by some other metabolism of the cell such as glucolysis. Whether the decrease in negative charge of the cell during glucolysis is such as to cause a general uptake of negative ions into the cell or whether the inward migration of glutamic acid is accompanied by an equivalent outward migration of some other negative ion has yet to be determined. Whatever the mechanism, it is clear that the metabolism of substances such as glutamic acid must depend upon the presence of an energy system. Thus Hills (1940) records no glutaminase action with washed suspensions of streptococci while McIlwain (1946) has found that washed suspensions of streptococci will show highly active glutaminase action if glucose is added to the cell-glutamine mixture. This has been confirmed by the author with the organisms used in the present work. McIlwain suggests a linkage between glucolysis and glutaminase action but, in view of the above work, it seems probable that glutaminase is an intracellular enzyme and that the glutamine cannot cross the cell wall and so gain access to the enzyme surface in the absence of a source of energy such as glucose. It is possible that micro-organisms have enzymes both on the outside of the cell and within the cell so that whether epi- or endo-metabolism of a given substrate takes place will depend on the presence of energy-giving substrates in the environment.

The concentration gradient across the cell membrane will have the effect that the bacterial cells are able to pick out amino-acids from an environment which is deficient in such nutrients. It would seem that this mechanism is more important from a biological point of view in nutritionally exacting organisms than in those which can synthesize their own amino-acids and are therefore independent of the external environment in this respect. It can be seen from Table 1 that there is a definite correlation, in the cases of lysine, glutamic acid, histidine and tyrosine, between the amounts of free amino-acid within the internal environment and the proportion of those amino-acids in the cell protein.

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The Assimilation of Amino-acids by Bacteria

2. The Action of Tyrocidin and some Detergent Substances in Releasing Amino-acids from the Internal Environment of *Streptococcus faecalis*

By E. F. GALE and E. SHIRLEY TAYLOR
Medical Research Council Unit for Chemical Microbiology,
Biochemistry Department, Cambridge

With a Note on Electron Micrographs of Normal and Tyrocidin-lysed Streptococci

By P. D. MITCHELL, *Biochemistry Department, Cambridge*
and G. R. CROWE, *Cavendish Laboratory, Cambridge*

SUMMARY: Treatment of *Streptococcus faecalis* cells containing high internal concentrations of lysine and glutamic acid with tyrocidin, Aerosol O.T., cetyltrimethylammonium bromide or phenol resulted in loss of the internal amino-acids. The substances used affected the cell wall so that the internal amino-acids leaked into the external environment; the rate of leakage was followed by a manometric method. The lytic action of tyrocidin, cetyltrimethylammonium bromide, Aerosol O.T., and phenol is sufficient to explain the disinfecting action of these substances.

It was shown (Gale, 1947) that lysine and glutamic acid passed into the internal environment of certain streptococci (Lancefield Group D) and that the internal concentration was markedly greater than that in the external environment at equilibrium. When the cells were removed from an amino-acid-rich medium and resuspended in distilled water or a suitable salt solution during 24–48 hr. at 4°, no loss of internal amino-acid occurred by diffusion. Hotchkiss (1944) stated that when bacterial cells were treated with tyrocidin or certain detergent substances a leakage of nitrogenous and phosphorus-containing substances occurred into the suspending fluid. It seemed that this might indicate that the action of tyrocidin, etc. was such that the amino-acids concentrated within the internal environment were released into the external environment and that the nitrogenous material estimated by Hotchkiss might consist partly of such amino-acids. The results reported in this paper show that this is the case (cf. Gale & Taylor, 1946).

METHODS

Organism and method of culture

The organism used was the same as that described previously (Gale, 1947). It was desirable to prepare cells which would contain a high internal concentration of lysine and glutamic acid; this can be done by growing the organism for approx. 12 hr. at 37° in medium A consisting of: casein digest; 0·1 % Marmite; 1·0 % glucose. The cells were washed once after harvesting.

Estimation of amino-acids. *l*-(+)-Lysine and *l*-(+)-glutamic acid were estimated manometrically by the use of specific amino-acid decarboxylase preparations (Gale, 1945).

Preparation of tyrocidin. Tyrothricin was obtained from the Wallerstein Co. through the courtesy of Dr R. Dubos and tyrocidin hydrochloride prepared therefrom by the method of Hotchkiss & Dubos (1941). The material was recrystallized once from methanol. We are indebted to Dr A. R. Trim of this department for samples of Aerosol O.T. and cetyltrimethylammonium bromide.

Antibiotic activities of tyrocidin, etc.

Table 1 shows the concentrations of the various antibiotics tested found necessary to prevent the growth of or to sterilize a culture of the group D *Streptococcus* used as test organism.

Table 1. *Inhibition of* Strep. faecalis *by antibiotic substances*

	Concentrations (mg./ml.)		
Antibiotic substance	Inhibition titre 10^6 cells/ml.	Sterilization of 10^8 cells/ml.	Cytolysis of 10^9 cells/ml.
Tyrocidin	0·001	0·1	1·0
Cetyltrimethyl-ammonium bromide	0·001	0·2	1·0
Aerosol O.T.	0·1	0·1	1·0
Phenol	2·5	10·0	30·0
Gramicidin	0·001	0·1	No effect
Patulin	0·1	20·0	No effect
Gentian violet	0·01	>2·0	No effect
Acriflavin	0·01	>2·0	No effect
Sulphathiazole	1·0	—	No effect
Penicillin	8 Oxford units	—	No effect

The inhibition titre in each case was determined by taking a series of tubes of medium A, adding the antibiotic in serial dilution at intervals such that each tube contained one-fifth the concentration of antibiotic of the previous one in the series, inoculating with a standard inoculum of 10^6 cells/ml. medium, and determining the limiting inhibitory concentration after 48 hr. incubation at 37°. In order to determine whether the action was bactericidal or bacteriostatic and also whether the inhibitory concentration varies with the number of cells present, a further test was carried out as a modification of the Rideal-Walker test. A 24 hr. culture of the organism in medium A containing approx. 10^8 viable cells/ml. was taken and serial dilutions of the antibiotics added as before to the complete culture, after 30 min. a loopful of culture was taken from each tube and streaked on to nutrient agar. The plate cultures were examined after 24 hr. incubation at 37° and the concentration of antibiotic necessary to sterilize the culture under these conditions noted; this value is given in Table 1 as the 'Sterilization of 10^8 cells/ml.' figure.

It can be seen from Table 1 that 1 μg. tyrocidin/ml. was sufficient to prevent the growth of the inoculum corresponding to 10^6 cells/ml., but 0·1 mg./ml. was necessary to sterilize a culture of approx. 10^8 cells/ml. It would appear that there is some quantitative relationship between the number of cells and the amount of tyrocidin necessary for their sterilization. The same was true for cetyltrimethylammonium bromide. In the case of Aerosol O.T. a concentration of 0·1 mg./ml. was required to sterilize either 10^6 or 10^8 cells/ml. although 10^9 cells/ml. required 1 mg. of Aerosol/ml. (see later). It is probable that this is a special case in which the physical state of the detergent substance is important and that no antibacterial activity is shown in solutions of Aerosol O.T. which are too weak for micelle formation to occur (Alexander & Trim, 1946). It is clear from Table 1 that the action of these substances is bactericidal in the concentrations quoted.

Compared with tyrocidin, phenol is a weak disinfectant, 0·25% being necessary to sterilize 10^6 cells/ml. and 1·0% for 10^8 cells/ml. Substances representative of other groups of antibiotics were also tested; gramicidin (Hotchkiss, 1944) and patulin (Raistrick, 1943) were both bactericidal towards the organism while gentian violet, acriflavin, sulphathiazole and penicillin were not bactericidal in concentrations 500 times the inhibition coefficient.

Effect on maintenance of internal free amino-acid concentration

When *Strep. faecalis* cells are grown in medium A for 12–14 hr. the cells contain a high internal concentration of lysine and glutamic acid in a free state and this internal amino-acid does not diffuse out of the cells if the latter are suspended in distilled water or salt solution at 4° (48 hr.) or 37° (6 hr.) (Gale, 1947). It is possible that some antibiotic substances may affect the cell wall in such a way that this is no longer the case. To test this, the organism was grown in medium A under optimum conditions for the production of a high internal concentration of free lysine and glutamic acid, the cells centrifuged out of the medium, washed once and a portion assayed for these amino-acids as previously described (Gale, 1947). Further portions of the cells were then suspended in distilled water to a final cell-suspension strength of approx. 5 mg./ml. (approx. 10^9 cells/ml.) and suitable concentrations of various antibiotic substances added. The suspensions were then incubated for 3 hr. at 37° after which the cells were again centrifuged out of suspension and the internal amino-acid content assayed again.

Table 2 shows that there was no significant loss of internal lysine or glutamic acid when the cells were suspended in distilled water but that the presence in the suspending fluid of tyrocidin, cetyltrimethylammonium bromide, or Aerosol O.T. (1 mg./ml.) or of phenol (10 mg./ml.) resulted in a complete disappearance of these amino-acids from the internal environment. The other antibiotic substances tested had no significant effect on the internal environment in this respect.

Manometric demonstration of lysis by tyrocidin, etc.

The results shown in Table 2 suggest that the action of tyrocidin and the detergent substances is to alter the permeability of the cell wall so that the free amino-acids in the internal environment leak into the suspending medium. That this is the case was shown as follows:

A thick washed suspension of *Strep. faecalis* cells grown in medium A was prepared and the internal lysine and glutamic acid content determined as usual on intact and boiled cells. One ml. of the suspension was then placed with 1 ml. buffer in the main compartment of a Warburg manometer cup fitted with two side-arms; into one side-arm 0·5 ml. of specific decarboxylase preparation in buffer was placed, and into the other 0·5 ml. of a suitable solution of tyrocidin or detergent substance. When the glutamic acid content was being studied M/5-acetate buffer pH 4·5 was used; with lysine, M/5-phosphate buffer at pH 6·0. After equilibration in a thermostat at 30°, the enzyme preparation was tipped into the main compartment and the 'external' amino-acid of the cell suspension assayed as usual (Fig. 1, curve 1). When the CO_2 evolution had ceased, after 10–15 min., the tyrocidin was added from the second side-arm.

Table 2. *Effect of antibiotic substances on maintenance of free amino-acid concentration in internal environment*

Cells suspended in distilled water containing substances as below for 3 hr. at 37° in suspension = approx. 5 mg. dry weight cells/ml. Dry weight of cells assayed = 52 mg.

	Amino-acid content of internal environment	
Suspending medium	Lysine (µl.)	Glutamic acid (µl.)
Initial content of cells	122	116
Distilled water alone	118	105
Tyrocidin 1 mg./ml.	0	0
Cetyltrimethylammonium bromide 1 mg./ml.	0	0
Aerosol O.T. 1 mg./ml.	0	0
Phenol 10 mg./ml.	0	0
Gramicidin 1 mg./ml.	120	111
Gentian violet 10 mg./ml.	110	56
Patulin 10 mg./ml. Acriflavin 10 mg./ml. Sulphathiazole 1 mg./ml. Penicillin, 8 Oxford units/ml.	No significant decrease over control in distilled water	

If the action of the tyrocidin is to liberate amino-acids from the internal environment of the streptococcal cells, this would be shown by evolution of CO_2, as the lysine or glutamic acid is attacked by the decarboxylase preparation. Fig. 1 shows the results obtained in such an experiment. The addition of 1·0 mg. tyrocidin at time 15 min. resulted in a rapid liberation of glutamic acid from the cells and the amount liberated was equal to that assayed in boiled cells (curve 2) and so corresponded to the total amount in the internal environment. The addition of smaller amounts of tyrocidin resulted in the liberation of correspondingly smaller amounts of glutamic acid: thus while 1·0 mg. tyrocidin gave rise to 100 µl. glutamic CO_2 in the case quoted, 600 µg. gave rise to 59 µl. and 300 µg. to 27 µl., suggesting a close quantitative relation.

The rate of evolution of glutamic CO_2 was much the same however much tyrocidin was added, and it was the final quantity that varied with the amount of tyrocidin added. It appears that the smaller amounts of tyrocidin released the internal amino-acid from a proportion of the cells only. To test this in another experiment 0·5 mg. tyrocidin was added to the cell suspension and the glutamic CO_2 evolution followed to cessation, when a further 0·5 mg. tyrocidin was added. Fig. 3 shows that the second addition causes the release of more glutamic acid from the cells, the two separate additions of 0·5 mg. have a combined effect equal to that of a single addition of 1·0 mg. The addition of further tyrocidin to the manometer in which 1·0 mg. had already been added had no further effect when the dry weight of cells present was of the order of 40–50 mg. Fig. 2 shows that similar results were obtained when the experiments were repeated using lysine decarboxylase instead of the glutamic enzyme.

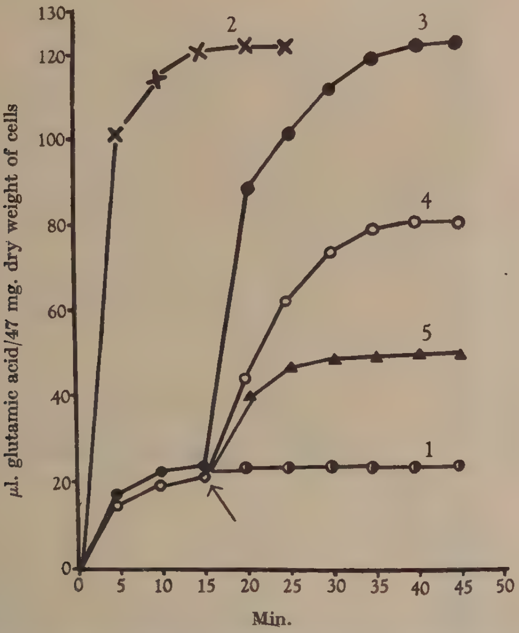

Fig. 1. Effect of tyrocidin on *Strep. faecalis*: liberation of glutamic acid from internal environment. Curve 1: glutamic acid assay of intact cells. Curve 2: glutamic acid assay of boiled cells. Curve 3: 1·0 mg. tyrocidin added at arrow 15 min. after addition of enzyme. Curve 4: 0·6 mg. tyrocidin added at arrow 15 min. after addition of enzyme. Curve 5: 0·3 mg. tyrocidin added at arrow 15 min. after addition of enzyme. Dry weight of cells assayed = 47·0 mg. Temperature = 30°. Manometer vessels contain initially 1·0 ml. M/5-acetate pH 4·5; 1·0 ml. washed suspension of *Strep. faecalis* cells (main compartment); 0·5 ml. glutamic decarboxylase (side-bulb 1); 0·5 ml. tyrocidin (side-bulb 2).

The weight of tyrocidin per cell needed for complete lysis in these experiments was of the same order as that shown in Table 1 for the amounts necessary to sterilize cultures. It seems probable that the lytic effect is sufficient to explain the antibiotic action of tyrocidin. From Figs. 1–3 it appears that while 1·0 mg. tyrocidin will lyse and sterilize 50 mg. streptococcal cells, 0·5 mg. will lyse only half these cells. To confirm that this was also true of the disinfecting action of tyrocidin, the experiment was repeated and viable counts carried out on (1) the untreated washed suspension, (2) the suspension treated with 1·0 mg. tyrocidin/50 mg. cells, and (3) the suspension treated with 0·3 mg. tyrocidin/50 mg. cells. Table 3 shows that whereas the larger amount of tyrocidin decreased the number of viable cells from 38×10^8 to 2×10^8, 0·3 mg. decreased the viable count by approx. one-third of this amount. It would seem from this evidence that about 10^8 molecules of tyrocidin are required to lyse and kill one *Streptococcus* cell.

Manometric experiments of this type were carried out with cetyltrimethylammonium bromide and Aerosol O.T. Very similar results were obtained with similar quantities, i.e. approx. 1 mg. of detergent substance lyses approx. 50 mg. cells. The rate of liberation of glutamic acid or lysine appeared somewhat slower with Aerosol O.T. than with the other substances, 100 µl. glutamic CO_2 being evolved after 20 min. after the addition of 1·0 mg. Aerosol O.T. compared with 10 min. for tyrocidin.

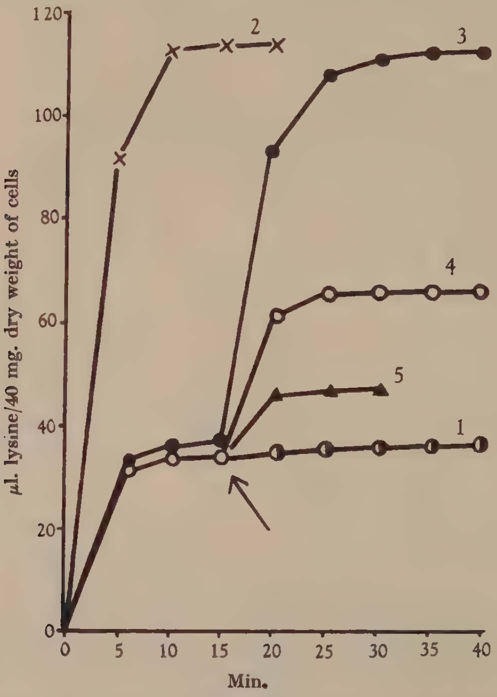

Fig. 2. Effect of tyrocidin on *Strep. faecalis*: liberation of lysine from internal environment. Curve 1: lysine assay of intact cells. Curve 2: lysine assay of boiled cells. Curve 3: 1·0 mg. tyrocidin added at arrow 15 min. after addition of enzyme. Curve 4: 0·5 mg. tyrocidin added at arrow 15 min. after addition of enzyme. Curve 5: 0·3 mg. tyrocidin added at arrow 15 min. after addition of enzyme. Dry weight of cells assayed = 40 mg. Temperature = 30°. Manometer vessels contain initially 1·0 ml. M/5-phosphate pH 6·0; 1·0 ml. washed suspension of *Strep. faecalis* cells (main compartment); 0·5 ml. lysine decarboxylase (side-bulb 1); 0·5 ml. tyrocidin (side-bulb 2).

The experiments quoted in Table 2 suggest that phenol in 1·0 % concentration has an effect on the cells similar to that described for the detergents mentioned. An attempt was made to test this by the manometric test as above. Fig. 4 shows the results obtained with glutamic decarboxylase on the addition of phenol to 66 mg. cells. The addition of 30 mg. phenol resulted in a slow evolution of glutamic CO_2, while 10 mg. phenol had a similar effect,

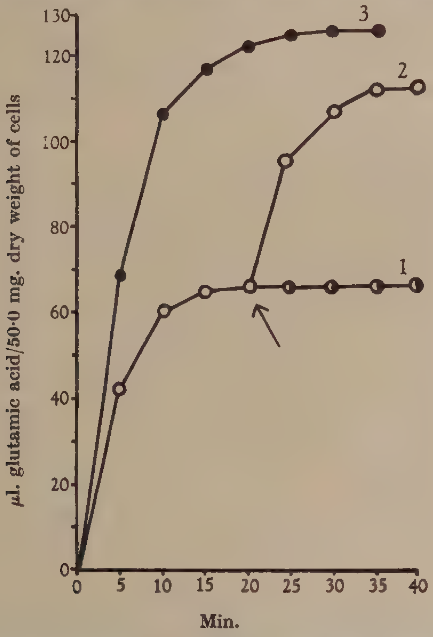

Fig. 3. Relation between quantity of tyrocidin added and amount of glutamic acid released from internal environment. Dry weight of *Strep. faecalis* cells assayed = 50·0 mg. Temperature = 30°. Manometer vessels made up with 1·0 ml. M/5-phosphate pH 6·0; 1·0 ml. washed suspension of *Strep. faecalis* cells, and 0·5 ml. glutamic decarboxylase (main compartment) with tyrocidin in side-bulbs. Manometers allowed to equilibrate and liberation of external glutamic CO_2 to cease before time = 0. Curve 1: 0·5 mg. tyrocidin tipped at time = 0. Curve 2: 0·5 mg. tyrocidin tipped at time = 0; further 0·5 mg. tyrocidin tipped at time = 20 min. (arrow). Curve 3: 1·0 mg. tyrocidin tipped at time = 0.

Table 3. *Effect of tyrocidin on viability of* Strep. faecalis

Strep. faecalis cells grown in medium A and made up into thick washed suspension (50 mg. dry weight/ml.). Tyrocidin added and left at room temperature for 30 min. before serial dilutions made for viable count.

	Viable count	
Cell preparation	Dilution 10^{-7}	Dilution 10^{-8}
Washed suspension 50 mg./ml.	425	38
Washed suspension 50 mg./ml. treated with 1·0 mg. tyrocidin/ml.	24	2
Washed suspension 50 mg./ml. treated with 0·3 mg. tyrocidin/ml.	220	26

giving a final evolution of CO_2 about one-fourth that produced by 30 mg. The slow rate of evolution is probably due to inhibition of the decarboxylase, as the enzyme is acting in the presence of 1·0 % phenol. It was not possible to carry out this type of experiment with lysine decarboxylase as this enzyme is more sensitive to phenol than the glutamic decarboxylase; the addition of the phenol is followed by a small evolution of CO_2 which may amount to 20–30 μl., after which the enzyme is inactivated. The results given in Table 2, however, leave no doubt that internal lysine is released from the cells. Phenol thus owes its disinfectant action to an effect upon the bacterial cell wall whereby essential constituents of the internal environment are released.

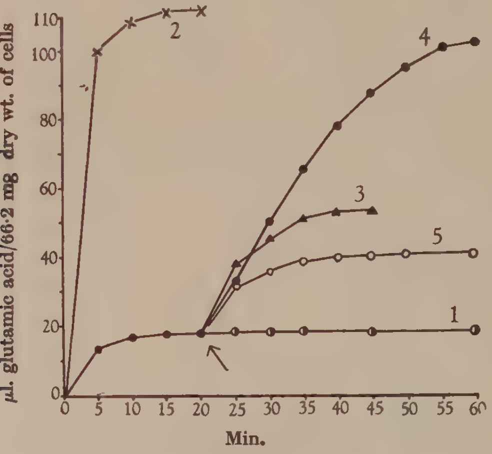

Fig. 4. Effect of phenol on *Strep. faecalis* cells: liberation of glutamic acid from internal environment. Curve 1: glutamic acid assay of intact cells. Curve 2: glutamic acid assay of boiled cells. Curve 3: 60 mg. phenol added at arrow 20 min. after addition of enzyme. Curve 4: 30 mg. phenol added at arrow 20 min. after addition of enzyme. Curve 5: 10 mg. phenol added at arrow 20 min. after addition of enzyme. Dry weight of cells assayed = 66·2 mg. Temperature = 30°. Manometer vessels contain initially 1·0 ml. N/5-acetate pH 4·5; 1·0 ml. washed suspension of *Strep. faecalis* cells (main compartment); 0·5 ml. glutamic decarboxylase (side-bulb 1); 0·5 ml. phenol (side-bulb 2).

Action on other cells. Experiments similar to those described above were carried out with washed suspensions of *Staph. aureus* and *Saccharomyces carlsbergensis*; the internal amino-acids of these cells were released by the action of tyrocidin in a manner essentially similar to that described above for streptococci.

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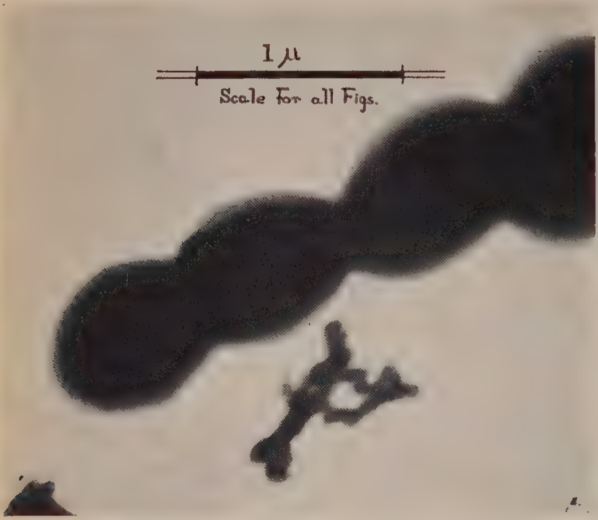

Fig. 1

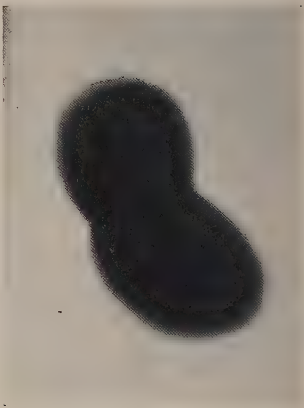

Fig. 2

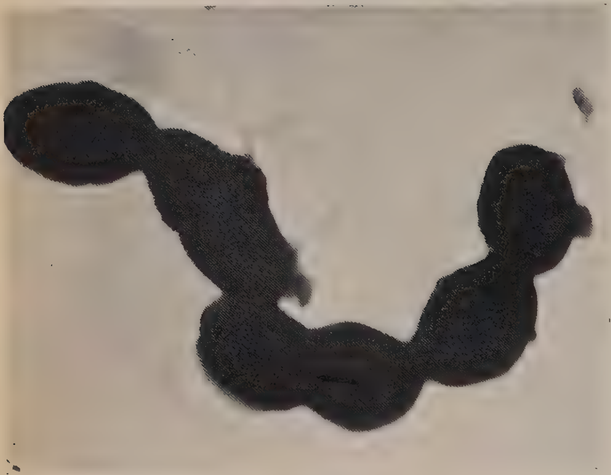

Fig. 3

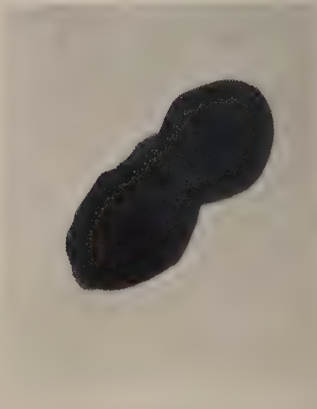

Fig. 4

E. F. GALE AND E. S. TAYLOR—CELL WALL DAMAGE, PLATE 1; P. D. MITCHELL AND G. R. CROWE—ELECTRON MICROGRAPHS OF NORMAL AND TYROCIDIN-LYSED STREPTOCOCCI

A Note on Electron Micrographs of Normal and Tyrocidin-lysed Streptococci

By P. D. MITCHELL and G. R. CROWE

Biochemistry Department and the Cavendish Laboratory, Cambridge

As shown in the foregoing the sterilization of streptococci (Lancefield Group D) by tyrocidin occurs concomitantly with the leakage of amino-acids from the internal environment. The leakage of the amino-acids is ascribed to changes in the barrier which normally confines them within the cell. It is of interest to consider the nature of this barrier and the site of action of the tyrocidin in disrupting it. Electron microscopy provides the most direct information.

Electron micrographs. A large number of normal and tyrocidin-treated streptococci were observed on the fluorescent screen of the RCA upright electron microscope at magnification 1800. Photographs were taken of the following preparations:

(i) Normal streptococci suspended in dust-free distilled water at a concentration of 20,000/cu. mm. for mounting on the collodion membrane. Pl. 1, figs. 1 and 2, show the normal cocci. The cell wall is very clearly seen as a cortical layer relatively transparent to the electron beam.

(ii) Streptococci treated with 1 mg. of tyrocidin/50 mg. dry weight and suspended in dust-free distilled water for mounting as above. Pl. 1, figs. 3 and 4 show the lysed cocci. The cell wall is completely disrupted.

The simplest hypothesis which may be proposed to account for the action of tyrocidin supposes that the amino-acid confining barrier is the cell membrane, and that this is disrupted by the entry of the highly surface-active tyrocidin. This cannot be the true mechanism, for the photographs show that tyrocidin does not disrupt only the cell membrane: the cell wall is disrupted. Presumably, either the cell wall is itself the amino-acid confining barrier, or, being attached to the cell membrane or some other confining structure, this is disrupted with it. In any case, it is certain that rupture of the cell wall is concomitant with the leakage of the amino-acids from the tyrocidin-treated cocci.

EXPLANATION OF PLATE

Fig. 1. Normal streptococci.

Fig. 2. Normal *Diplococcus*.

Fig. 3. Tyrocidin-lysed streptococci. Note the cell wall trailing away in some places. Compare Fig. 1.

Fig. 4. Tyrocidin-treated *Diplococcus*. Note the cell wall gathered into cleavage furrow. Compare Fig. 2.

The Assimilation of Amino-acids by Bacteria

3. Concentration of free Amino-acids in the Internal Environment of Various Bacteria and Yeasts

By E. SHIRLEY TAYLOR

Medical Research Council Unit for Chemical Microbiology,
Biochemistry Department, Cambridge

SUMMARY: Sixteen Gram-positive organisms (including three yeasts) were able to assimilate glutamic acid and lysine from the external medium and to concentrate these amino-acids in the internal environment, while eleven Gram-negative organisms were unable to do so.

It has been shown (Gale, 1947) that *Streptococcus faecalis* cells possess a high concentration of certain amino-acids existing in a free state within the cells, the internal concentration of lysine and glutamic acid being much greater than that in the external environment in equilibrium with the internal environment. Lysine is able to diffuse into the cell under certain conditions, while the migration of glutamic acid across the cell wall appears to be a process requiring energy, obtainable from fermentation processes. The gradient in concentration of lysine and glutamic acid across the cell wall appears to be maintained by properties of the cell wall itself, since rupture of the cell wall with tyrocidin, etc. results in the release of the internal amino-acids (Gale & Taylor, 1947). A biological consequence of this gradient across the cell wall is that the cell is able to select and concentrate certain amino-acids from a deficient medium. This mechanism should be of greater importance to organisms which are nutritionally exacting with regard to amino-acids than to organisms which can synthesize their amino-acid requirements (Gale, 1947). The present communication deals with the distribution amongst various bacterial genera and species of this cell wall gradient effect, as judged by the presence of free amino-acids in the internal environment of the cells.

EXPERIMENTAL

Organisms. The majority of the organisms listed in Table 1 were isolated by members of this Unit but we are indebted to Dr H. McIlwain for the strain of *Strep. haemolyticus* 'Richards', to Dr P. M. F. Shattock for strains of lactobacilli and the Cambridge Pathology Department for *Neisseria catarrhalis* and *Bacterium aerogenes*. The three yeasts were obtained from the Carlsberg Laboratory. Organisms obtained from or deposited in the National Collection of Type Cultures are indicated in Table 1 by their catalogue numbers.

Growth media. All organisms were grown on a basal medium consisting of tryptic digest of casein with the addition of glucose (2·0 % for coliform organisms and clostridia; 1·0 % for lactobacteriaceae); 0·1 % Marmite was added to

Table 1. *Free amino-acids in the internal environment of various organisms*

Organism	Gram-stain reaction	Time of growth (hr.)	Temp. of growth (° C.)	µl. amino-acid/100 mg.				
				Arginine	Glutamic	Histidine	Lysine	Tyrosine
Yeast foam	+	40	25	134	150	75	223	20
Dutch top yeast	+	40	25	187	378	64	543	30
Saccharomyces carlsbergensis	+	40	25	55	198	30	165	7
Lactobacillus casei, YCT1	+	40	28	0	174	0	97	0
L. delbruckii, B	+	40	37	19	80	—	103	—
L. helveticus, B	+	40	37	—	74	8	70	—
Strep. faecalis, ST	+	16	37	0	230	10	309	—
Strep. faecalis, SF	+	16	37	6	107	—	110	—
Strep. haemolyticus 'Richards'	+	16	37	0	99	—	83	—
Staph. aureus, A	+	16	37	0	293	—	59	—
Staph. aureus, D	+	16	37	0	456	—	99	—
Sarcina lutea	+	56	25	6	225	16	61	0
Micrococcus lysodeikticus	+	24	37	0	203	—	108	—
Cl. sporogenes	+	14	37	0	81	—	31	—
Cl. septicum, P3 (547)	+	14	37	0	7	—	36	—
B. mesentericus	+	14	37	0	14	—	70	—
B. subtilis	+	14	37	0	19	—	29	—
N. catarrhalis	–	24	37	0	0	0	0	0
B. brevis	–	14	37	0	0	—	0	—
Bact. coli (86)	–	14	37	0	0	0	0	0
Bact. coli Taylor	–	14	37	0	0	—	0	—
Bact. coli (7020)	–	14	37	0	0	—	0	—
Organism N.C.T.C. No. 6578	–	14	37	0	0	0	0	0
Bact. aerogenes I	–	14	37	0	0	—	0	—
Bact. aerogenes II	–	14	37	0	0	—	0	—
Proteus vulgaris	–	14	37	0	0	—	0	—
Pseudomonas pyocyanea, G138	–	14	37	0	0	—	0	—

media used for lactobacteriaceae and 0·5 % glutamine for *Strep. haemolyticus* 'Richards'. The media were dispensed in flasks for coliforms, streptococci, lactobacilli and clostridia, conditions being made strictly anaerobic for lactobacilli and clostridia; for all other organisms the medium was dispensed in Roux bottles lying flat so as to expose a large surface to the air during growth. Yeasts were grown in Roux bottles containing Stephenson's inorganic medium (1939) with the addition of 0·2 % 'Difco' yeast extract and 4 % glucose, the pH being adjusted initially to 6·0.

Estimation of amino-acids. Amino-acids were estimated by the use of specific amino-acid decarboxylase preparations (Gale, 1945, 1946). The free amino-acids in the internal environment of the cells were estimated by the method of Gale (1947). To avoid respiratory gas changes with intact cells, manometers were filled with nitrogen during assay.

Estimation of the dry weight of cells. The dry weight per ml. of cell suspension was estimated turbidimetrically by the use of the Hilger photoelectric absorptiometer, calibrated for each of the organisms used by drying a known volume of thick suspension of known turbidity to constant weight.

Method. The cultures were incubated until it was judged that active cell division had just ceased. The organisms were then centrifuged out of the medium, washed once with distilled water and made up into thick suspension of which the dry weight was determined. The internal amino-acid contained in 1 ml. of the cell suspension was then estimated by comparison of the amino-acid content of the suspension before and after boiling the cells (Gale, 1947). For comparative work, the results are expressed as μl. amino-acid in the internal environment of 100 mg. dry weight of cells.

RESULTS

Table 1 shows the amounts of arginine, glutamic acid, histidine, lysine and tyrosine found in the internal environment of the cells of various species of micro-organisms. The tyrosine and histidine values were generally small and were carried out in a few cases only. No arginine was found in the majority of bacterial species tested, many of which are known to possess enzymes which attack the arginine molecule (Hills, 1940). The three yeasts tested all had a high internal concentration of arginine. An interesting correlation can be observed between the presence of lysine and glutamic acid in a free state in the internal environment of the cells, and a positive reaction to the Gram-stain. None of the eleven Gram-negative organisms tested show any internal free amino-acids, whereas all sixteen Gram-positive organisms show internal concentrations of lysine and glutamic acid.

The external medium in all cases consisted essentially of a tryptic digest of casein in which the amounts of free lysine and glutamic acid varied from 100–200 μl./ml.; the histidine content was *c.* 20–30 μl./ml. As found by Gale (1947) 100 mg. *Strep. faecalis* cells possessed an internal environment of volume 0·27–0·34 ml. Consequently, if the concentration of free amino-acid within the cell is the same as that in the external environment, then the amounts of lysine and glutamic acid within the cell would be of the order 30–70 μl./100 mg.

or of histidine 5–10 μl./100 mg. Gale (1947) showed in the case of *Strep. faecalis* that the internal concentrations of lysine and glutamic acid within the cell were considerably greater than those outside, whereas histidine appeared inside the cell in approximately the same concentration as outside. Assuming that these approximate calculations hold true for other bacteria, it is possible to decide whether the amounts of free amino-acid estimated in the internal environment represent an increase of concentration over those existing in the external medium during growth. Inspection of the values in Table 1 shows that the glutamic acid content of the Gram-positive organisms with the exception of *Clostridium septicum*, *B. subtilis* and *B. mesentericus*, showed an internal concentration higher than that in the external medium. In the case of lysine, exceptions may occur with *Staph. aureus* A., *Sarcina lutea*, *Cl. septicum*, *Cl. sporogenes* and *B. subtilis*.

The results suggest that the Gram-positive organisms possess a cell wall which is permeable to amino-acids only under certain conditions as described by Gale (1947) for *Strep. faecalis*. The Gram-negative species do not show this effect.

The question arises whether the Gram-negative cells are completely permeable to amino-acids, in which case they would have an internal concentration of amino-acid equal to that of the medium at harvesting. On washing prior to assay, this amino-acid would be lost in the washing water. To test this a thick suspension of *Bact. coli* cells was prepared as usual and suspended in a solution of lysine containing 200 μl. lysine/ml. After 30 min. the cells were centrifuged down and an attempt made to estimate the lysine in the supernatant fluid and in the packed cell mass. The cell mass was then re-suspended in water, centrifuged down, the supernatant liquid collected, evaporated to small bulk *in vacuo* and assayed for lysine. No evidence could be obtained that the cells had carried down any lysine from the solution other than that mechanically entangled with the cell mass and the washings from the cells were devoid of lysine. These results appear to suggest that the Gram-negative cells are unable to take up amino-acids in a free state into their internal environment.

DISCUSSION

The results above show that there is a clear-cut distinction between Gram-negative and Gram-positive bacterial species in that the latter possess an internal environment in which free amino-acids exist sometimes in a concentration considerably greater than that in the external environment at equilibrium, while the former do not show this property. The effect in *Strep. faecalis* depends partly upon the properties of the cell wall and partly on the existence of a negative charge inside the cell (Gale, 1947). Henry & Stacey (1943) have demonstrated that the reaction to the Gram stain depends upon the presence in the Gram-positive species of a magnesium ribonucleotide in the surface layers of the cell which combines with protein, and confers upon the nucleoprotein so formed the property of combining with the violet dye used in the staining technique. The present studies show that a further difference lies in the properties of the cell walls of the two groups such that the Gram-positive group can assimilate free amino-acids into the cell and concentrate them in the internal environment. The Gram-positive organisms are, in general, more exacting in their amino-acid requirements than Gram-negative species and the concentration gradient existing across the cell wall must be of biological importance in facilitating the assimilation of the essential amino-acids.

The author is indebted to the Medical Research Council for a personal grant.

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The Toxins of *Clostridium oedematiens* (*Cl. novyi*)

By C. L. OAKLEY, G. HARRIET WARRACK and
PATRICIA H. CLARKE

The Wellcome Physiological Research Laboratories, Beckenham, Kent

SUMMARY: Six antigenic components have been identified in toxic filtrates from *Clostridium oedematiens*, which have been designated α, β, γ, δ, ϵ and ζ.

α is the classical lethal toxin of *Cl. oedematiens* and β and γ are haemolytic lecithinases, β being certainly necrotizing; δ and ζ are haemolytic, δ being oxygen labile; and ϵ is probably responsible for pearly layer formation. Type A strains of *Cl. oedematiens* produce α, γ, δ and ϵ; type B strains α, β and ζ; type C strains produce none of these components when examined by our methods. Two strains of *Cl. haemolyticum* produced the β lecithinase.

By methods based on certain characteristic properties of these six antigenic components, 'oedematiens' antisera can be tested for the corresponding antibodies.

In the identification of the various types of *Cl. oedematiens*, methods based on the properties of the β, γ and ϵ components give more consistent and clear-cut results than those depending on morphology, colonial formation or fermentation reactions.

In 1894 Novy described as *Bacillus oedematis maligni* no. 2 a Gram-positive obligate anaerobic bacillus isolated by him from guinea-pigs inoculated with milk nuclein; this account is generally regarded as the first reference to the organism now known as *Clostridium oedematiens* (*Cl. novyi*, Bergey, Breed, Murray & Hitchen, 1939). Novy showed that injection of pure cultures of the organism led to death of experimental animals, and observing that death was not necessarily associated with evidence of local or general growth of the organism, concluded that death was due to injected toxic products. Since that time numerous accounts have appeared of organisms related to *Bacillus oedematis maligni* no. 2. In 1934 Scott, Turner & Vawter divided them into three groups as shown in Table 1.

Products detected in culture filtrates

The lethal toxin. Filtrates from *Cl. oedematiens* type A and type B often contain a lethal toxin, which is thermolabile (Zeissler & Rassfeld, 1929), produces on injection into animals the gelatinous pink-stained oedema of connective tissue and muscle characteristic of *Cl. oedematiens* infections (Weinberg & Séguin, 1915*a*, *b*, *c*; Menk, 1931/2), produces a local lesion in the rabbit cornea (Gildemeister & Schlossberger, 1932) and is readily neutralized by anti-oedematiens sera produced in animals by injection of either type A or type B filtrates (Turner & Davesne, 1927*a*, *b*). All these activities are ascribed, probably correctly, though on little evidence, to a single lethal toxin; methods for its routine production were given by Walbum & Reymann (1937).

Haemolysins. Both *Cl. oedematiens* type A (Menk, 1931/2; Celarek & Stetkiewicz, 1936; Reed, Orr & Baker, 1939) and type B (Turner & Davesne, 1927*b*; MacEwen, 1931) produce haemolysins, and the literature contains evidence that some of these are distinct from the lethal toxin and that the type A haemolysin complex is different from that produced by type B. Glotowa, Ostrowskaya, Ssilanowa & Milaschewskaya (1934) found poor agreement between lethal and haemolytic values for sera against *Cl. oedematiens* filtrates. Reed & Orr (1941) state that for the most part haemotoxins as well as lethal toxins of the pathogenic species of *Clostridium* are species-specific, but give no evidence to support this view. Keppie (1944) showed that type A antisera failed to neutralize the haemolysin of *Cl. oedematiens* type B filtrates, but did not determine whether this was dependent on their strength, or whether the haemolysins were antigenically distinct. Hayward & Gray (1946), devising an empirical haemolysin test to differentiate between *Cl. oedematiens* and *Cl. septicum*, found that sera neutralizing the haemolysins of *Cl. oedematiens* (type A) failed to neutralize those of *Bacillus gigas* (*Cl. oedematiens* type B).

Table 1. *Classification of* Cl. oedematiens (*Scott* et al. 1934)

Type	Organisms included	Origin	Size	Fermentation of glycerol	Experimental pathogenicity	Toxigenicity
A	*B. oedematis maligni* no. 2 (Novy, 1894) *B. oedematiens* (Weinberg & Séguin, 1915*b*) *Cl. oedematiens* *Cl. novyi*	Gas gangrene of man and other animals	Small 0·8–1 × 2·5–5 μ	+	+	+
B	*B. oedematiens* (Albiston, 1927; Turner & Davesne, 1927*a*, *b*) *B. gigas* (Zeissler & Rassfeld, 1929)	Black disease and Bradsot of sheep	Large 1·2–2 × 10–14 μ	−	+	+
C	Bacillus of osteomyelitis bacillosa bubalorum (Kranefeld & Djaenoedin, 1933, 1936)	Bacillary osteomyelitis of buffaloes	Large 1·5–2 × 8–10 μ	−	−	−

Substances affecting egg-yolk emulsion (lecitho-vitellin, L.V.). Crook (1942) claimed that many strains of *Cl. oedematiens* produced opalescence in egg-yolk emulsion (see Macfarlane, R. G., Oakley & Anderson, 1941) and that some strains produced as intense an opalescence as that due to filtrates of *Cl. welchii* type A. Macfarlane, M. G. (1942) stated that some *Cl. oedematiens* filtrates (probably derived from the type B strain 'Albiston') contained a weak lecithinase antigenically distinct from the lethal toxin and from the lecithinase (α-toxin) of *Cl. welchii*. Small amounts of a lecithinase are extractable from the tissues of animals infected with *Cl. oedematiens* (type not stated; McClean, Rogers, Williams & Hale, 1943). Nagler (1945) states that neutralization tests on plates show that the substances diffusing from *Cl. oedematiens* colonies and producing opalescence on egg-yolk agar and haemolysis on sheep-blood agar are probably identical.

The substance producing the 'pearly layer'. Nagler (1944, 1945) has shown that the surface of colonies of *Cl. oedematiens* type A growing on egg-yolk sheep-blood agar is covered by a 'pearly layer' easily detachable from the surface, and that types B and C do not produce this effect. The substance giving rise to this effect is rather labile, thermolabile, and was not neutralized by two anti-oedematiens sera tested. McClung, Heidenreich & Toabe (1946) have claimed that *Cl. oedematiens* type A can be distinguished from type B and from *Cl. sordellii* and *Cl. perfringens* by the 'reactions' produced on a medium containing 4% proteose-peptone, glucose, egg yolk and defibrinated rabbit blood; these reactions are said to be distinct and to be inhibited by 'appropriate' sera; it is stated that the blood may be omitted. No other details are given.

Hyaluronidase. Of fifteen strains of *Cl. oedematiens* examined by McClean *et al.* (1943) only seven produced measurable amounts of hyaluronidase; Keppie's thesis (1944) shows that these were all type B strains.

In the present paper evidence is offered for the existence in *Cl. oedematiens* filtrates of at least six antigenic substances, by the use of which, and of suitable neutralizing sera, it is easy to distinguish between *Cl. oedematiens* types A and B and other anaerobes.

EXPERIMENTAL

Material and methods

Strains. We used 103 strains of *Clostridium*, either described by other workers as *Cl. oedematiens*, *Cl. novyi* or *B. gigas*, or regarded by them as more closely related to these organisms than to any other *Clostridium* so far described. All these were compared and as far as possible classified on a basis of morphology, colony form, fermentation reactions and toxin production.

The type A strains were of the usual origin: two from gas gangrene in animals, forty-three from gas gangrene in man, including the classical strains 'Cossard', 'Domange', 'Jolly', and 'B 140' from the 1914–18 war, and thirty-nine from various battle-fronts (three from Germany), including air-raid casualties in the war of 1939–45.

Of the sixteen type B strains twelve were derived from sheep, five from cases of Black disease in Australia, three from England and four from Germany. The remaining four were isolated from man, three from mixed infections; the fourth was the only organism recovered from a typical fatal case of gas gangrene (MacLennan, unpublished).

Only two type C strains were available.

Cultural methods. Cultures were grown for toxin production on the medium used for routine production of lethal toxin. Before 1943 this was Wright's broth prefermented with *Aerobacter aerogenes*; since then the medium has been a papain digest of horse meat (total N 6–7 g./l.) to which was added 10% of a sat. sodium sulphate solution extract of horse muscle (Macfarlane & Knight, 1941). Sodium thiolacetate or sodium 'hydrosulphite' (dithionite) was added to ensure anaerobic conditions. Cultures were grown in 1 or 4 l. bottles, occasionally in smaller volumes; bacteria were removed from the culture fluid either by centrifugation or by filtration through paper after mixing with 'Hyflo-supercel' (Johns-Manville, Ltd., 5 g./l.). When required sterile a filtrate was refiltered through Berkefeld filters, or through Seitz sterilizing pads, filtering at least 30 ml./sq.cm. of the pad.

For some purposes filtrates were precipitated by saturation with ammonium sulphate and the precipitate dried *in vacuo* over silica gel or dialysed overnight against tap water. If the dried precipitate contained too much ammonium sulphate for easy use, the powdered material was stirred up with dry chloroform; much of the ammonium sulphate sank to the bottom, and the floating 'toxin' was skimmed off and dried.

Sera. We examined forty sera, obtained at all stages of immunization from horses hyperimmunized against *Cl. oedematiens*. Of all these sera the anti-lethal values (i.e. the values obtained by subcutaneous injection of lethal-toxin-antitoxin mixtures into mice) were accurately known.

The general method of titration has been to devise indicators of toxic activity, choose arbitrary standard sera active against filtrates affecting these indicators, and to estimate the values of other sera against these standards. To do this the test dose of filtrate was determined as that amount of filtrate which, when mixed with a convenient volume of standard serum (arbitrarily called x units), allowed to stand for an appropriate time and added to the indicator, produced a standard indicating effect. The values of sera were then determined by estimating the amount of serum required to neutralize the test dose of filtrate to the point at which it just produced the standard indicating effect on the indicator. Such a mixture contains x units of antitoxin; from this information the value of any serum is readily derived. All filtrates were also examined by flocculation and by animal tests for the classical lethal toxin.

EXAMINATION OF FILTRATES FOR TOXINS

Lecitho-vitellin (*L.V.*) *tests*

Level of testing three units; diluent M/5-sodium acetate-acetic acid to pH 6·5 (=S.A.B.); allow mixture to stand ½ hr., add 0·5 ml. L.V. (Macfarlane, R. G. *et al.* 1941) as indicator, place tests in water bath at 37° for 1 hr., read following morning; slight opalescence was taken as the indicating effect.

The toxic filtrates from *Cl. oedematiens* type A available to us when we began this work readily produced opalescence in egg-yolk emulsions (L.V.); we therefore chose serum R7903 as standard, gave it a value of 1700 units and determined the values of our other sera against six different type A filtrates. Strictly speaking R7903 and Ex949 (see p. 97) are secondary standards, the values of which are given in terms of our original arbitrary standards, now exhausted. Table 2 shows the results obtained from a representative selection of the sera.

Table 2. *Comparison of anti-lethal values of anti-oedematiens sera with lecitho-vitellin values against several different* Cl. oedematiens *type A filtrates*

		Lecitho-vitellin value against filtrate			
Serum	Anti-lethal value	AE 51	AE 208	AE 327	OC 19345
R7903*	950	1700	1700	1700	1700
9517	1700	800	800	900	700
9783	1000	740	660	1100	700
202	800	670	580	700	750
644	800	630	630	800	500
R7548	550	270	270	270	160
171	1·7	290	240	250	—

* Standard.

Two conclusions can be drawn from Table 2. First, that whatever L.V. values are chosen for comparison, they bear no relationship whatever to the anti-lethal values; it is probable therefore that whatever is producing opalescence in L.V. is not the lethal toxin, a view which is supported by the fact that *Cl. oedematiens* filtrates capable of producing dense opalescence in L.V. may show no lethal activity. Secondly, though the L.V. values of sera agree fairly well among themselves, there are occasional very large discrepancies (cf. values of R 7548 for instance). Such discrepancies are most readily explained by the existence in the filtrates of varying proportions of more than one substance affecting the indicator (egg-yolk emulsion).

Haemolytic tests

Level of testing 1–3 units; diluent S.A.B.; allow mixtures to stand ½ hr., add 0·5 ml. 6 % washed horse red cells in saline, place tests in water bath at 37° for 1 hr., examine on removal from bath, and not less than 2 hr. thereafter. Indicating effect 20 % haemolysis. Rabbit cells may be used; sheep cells are rather insensitive.

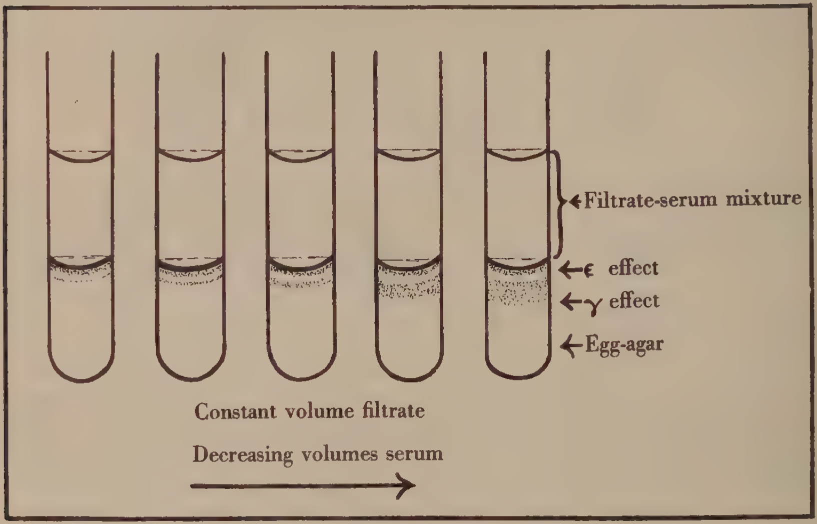
Table 3. *Comparison of haemolytic and lecitho-vitellin values of sera against two different* Cl. oedematiens *type A filtrates*

	Haemolytic values against		Lecitho-vitellin values against	
Serum	AE 327	OC 19345	AE 327	OC 19345
R 7903	1700	1700	1700	1700
202	650	800	700	750
850 B	650	800	300	160
644	950	850	800	500
9783	1100	1100	1100	700
171	1800	—	250	—

Haemolytic tests using the same standard filtrates and sera (Table 3) support the view that two independent antigens are present, for though for both the filtrates shown the haemolytic and L.V. values of some of the sera (e.g. 202) agree, for others the L.V. value against one or other filtrate is lower than the haemolytic value. The haemolytic values against all the filtrates showed good agreement, suggesting that only one haemolytic substance was present. Moreover, when the L.V. value differs from the haemolytic value, the L.V. values using different filtrates show some indication of being in proportion; thus for sera 644, 9783, 850 B the L.V. values against filtrate OC 19345 are about half those against filtrate AE 327. We therefore conclude that the type A filtrates examined by us contained two substances distinct from the lethal toxin (α), one causing opalescence and haemolysis (γ), the other opalescence only (ϵ).

Further evidence in favour of this view was obtained from partial neutralization tests; for instance if a filtrate were partially neutralized with a serum whose L.V. value was much lower than its haemolytic value (i.e. a serum containing as compared with the standard proportionately more γ-antitoxin than ϵ-antitoxin) it might be expected that titratable ϵ would remain after all the γ had been neutralized, and that using the partially neutralized mixture as test toxin, L.V. tests would give the L.V. value for sera when it was lower than the haemolytic value (using a serum like 171 as standard) and a value higher than either when the haemolytic and L.V. values were equal. Though these expectations have on the whole been fulfilled, practical difficulties due to the poor neutralizing power of our sera, and the small number of indicating doses of ε present in most filtrates, make the results rather unreliable.

Another method of testing the independence of γ and ε has been derived from the experiments of Nagler (1944, 1945), who showed that if *Cl. oedematiens* type A was grown on egg-yolk sheep-blood agar plates a pearly layer was formed on the surface of the colony. We had found (like McClung *et al.* 1946) that the blood could be omitted from the medium; when this was done, not only could the pearly layer be seen at the surface, but another more diffuse opalescence could be seen below it. When blood is included in the medium, a haemolytic zone of roughly the same extent as the diffuse opalescence is formed round the colony. All these observations raised our hopes that the pearly layer might be due to ε, and the diffuse opalescence and haemolysis to γ. If this were true we might be able to achieve physical separation of the γ and ε effects in L.V. tests. We therefore devised the egg-agar test (Text-fig. 1).



Text-fig. 1. The egg-agar test.

Filtrate serum mixtures are made in the usual way, allowed to stand ½ hr., then poured on the top of a column of Weinberg's V.F. agar containing about 5 % of L.V., and the whole incubated in the water-bath at 37° for 18–24 hr. If the filtrate-serum mixture is under-neutralized two distinct effects are produced in the agar: at the surface a dense opalescence, and deeper in the medium and separated from the surface opalescence by a comparatively clear zone, a faintly opalescent ring. If the latter opalescent ring is used as indicator in serum-titration tests, 'egg-agar' values can be obtained for sera; and these (as Table 4 shows) agree with the haemolytic values whether the L.V. values agree with them or not. There is therefore considerable evidence that γ is haemolytic and opalescence-forming, as distinct from ε, which causes only opalescence. Attempts to carry out anti-ε tests on sera, using the surface opalescence as indicator either in tubes or on egg-agar plates, have been successful only on occasion, as the end-point of such titrations is far too uncertain for accuracy.

Table 4. *Comparison of lecitho-vitellin, haemolytic and egg-agar values of sera against the same* Cl. oedematiens *type A filtrate* (OC19345)

	Indicator		
Serum	Lecitho-vitellin	Red cells	Egg agar
R7903	1700	1700	1700
R7548	160	300	310
850B	160	800	900
9559	350	400	400
644	500	850	850
645	500	800	750
9783	700	1100	1200
202	750	800	800

Type A filtrates from some cultures in peptic digest media contain little γ as judged by L.V. tests; they may, however, be vigorously haemolytic. γ-, like the α-toxin of *Cl. welchii*, is a hot-cold haemolysin; filtrates from peptic digests may haemolyse almost immediately at 37°. The haemolysin (δ) is oxygen-labile, and is to some extent neutralized by heterologous sera. As Table 1 shows, serum values against this haemolysin (diluent phosphate buffer pH 6·5 + M/25-sodium thiolacetate, indicator 0·5 ml. 6 % washed horse cells in saline) show no relationship to the α, γ or ε values; δ is therefore probably distinct from α-, γ- and ε-toxins.

Table 5. *Comparison of anti-δ with other values of sera, to show independence of δ-toxin*

Serum	Anti-α value	Anti-γ value	Anti-ε value	Anti-δ value
R7548	550	270	160	20
645	775	800	500	90
202	800	700	>700	50
644	800	700	550	180
28	950	700	400	*c.* 5
R7903	950	1700	>1700	30
9559	1370	400	350	80
9517	1700	1000	750	60

At this point we had to abandon the investigation for a year or so. When we returned to it we found that filtrates provided for us were no longer neutralized in haemolytic or L.V. tests by our old well-tried sera; nor was the new activity due to α-toxin. Investigation showed that these new filtrates were derived from type B strains, and that these filtrates were now being used to immunize horses. New sera from these horses were obtained and tested against a new standard Ex949 (280 units) in L.V. and haemolytic tests. Consistent values were obtained in L.V. tests against several type B filtrates (Table 6), suggesting that only one substance affecting L.V. is present in these filtrates; in haemolytic tests results agreeing with the L.V. results were often obtained; the new 'opalescing' substance (β) is probably haemolytic. Against some filtrates, however, haemolytic values considerably less than the L.V. values are obtained (Table 7); this may be due to the presence in the filtrates of a haemolysin antigenically distinct from β. Filtrates suitable for investigating this point are not yet available. The few sera containing antibody to both β- and γ-toxins (e.g. 850A) were from horses immunized with filtrates from both types A and B strains. Like γ, β is a hot-cold lysin.

Table 6. *Comparison of anti-α and anti-γ values of sera with their lecitho-vitellin values against several* Cl. oedematiens *type B filtrates*

Serum	Anti-α value	Lecitho-vitellin values against filtrate OC91044	OC271144	OC15145	OC23445	Anti-γ value
Ex949*	1200	230	230	230	230	50
2705	1650	200	180	160	170	3
T61	1650	450	460	400	500	120
2209	1675	240	330	180	200	12
850A	1750	1200	1300	1600	1400	750
T3	6250	2150	3000	3000	2000	120

* Standard.

Table 7. *Comparison of lecitho-vitellin, necrotizing and haemolytic values of sera against* Cl. oedematiens *type B filtrates*

Serum	Lecitho-vitellin values	Necrotizing values	Haemolytic values against filtrate OC91044	OC271144	OK15145
Ex949	230	230	230	230	230
T61	450	300	350	400	<50
T3	2500	2000	2200	2800	<50
2209	250	230	300	300	90
850A	1400	1100	1300	1400	1000

It may be noticed that the letters assigned to the antigens do not apparently correspond to the order of discovery. This is because we consider that M. G. Macfarlane's description of a lecithinase (almost certainly derived from a type B strain of *Cl. oedematiens*) is adequate to define this toxin, here therefore assigned the letter β. The haemolysin δ was established before the differentiation between γ and ϵ was satisfactorily proved.

At this point we sent samples of filtrates containing β- and γ-toxins to Miss M. G. Macfarlane (Lister Institute, Chelsea), who showed that both liberated water-soluble phosphorus from lecithin, and were therefore probably lecithinases; she also confirmed our view that they were antigenically distinct, making use of the method for titrating sera by enzyme inhibition devised for lecithinases by Macfarlane & Knight (1941).

The fact that β and γ are lecithinases affords other indicators for them, as in virtue of this activity they might be expected not only to be haemolytic, but to produce a necrotic lesion on intradermal injection, or death on intravenous injection. No filtrate containing γ-toxin in which all the α-toxin had been neutralized with sera containing no γ-antitoxin had any lethal or necrotizing power, presumably because so little active γ-enzyme was present; several β-containing filtrates in which the α-toxin had been neutralized by sera containing no β-antitoxin were strong enough to produce a necrotic lesion, but not to kill with certainty. Serum value tests were therefore put up against the standard, and after standing half an hour 0·2 ml. of the filtrate-serum mixture was injected intradermally into depilated guinea-pigs. The indicating effect was taken as a necrotic lesion 2 × 2 mm. developing in 2 days. The serum values so obtained indicate that β-toxin is necrotizing as well as haemolytic (Table 7).

Some filtrates from type B strains after neutralization of all β-toxin (as judged by failure of the mixture to affect L.V.) were still haemolytic. This haemolysin (ζ), though not definitely oxygen-labile, was neutralized to some extent by heterologous sera, and as the serum values against it show (Table 8) is distinct from α-, β-, γ-, δ- and ϵ-toxins.

Table 8. *Comparison of various values of sera against* Cl. oedematiens *filtrates, to show independence of ζ-toxin*

Serum	Anti-α value	Anti-β value	Anti-γ value	Anti-δ value	Anti-ϵ value	Anti-ζ value
202	800	0·5	700	50	> 700	30
644	800	2	700	180	550	140
9783	1000	1	1100	20	700	70
9559	1370	5	400	80	350	15
Ex 949	1200	230	50	800	15	1000
T 61	1650	450	65	10	10	150
T 3	6250	2500	120	8	12	110
850 A	1750	1400	750	220	180	5000
R 7903	950	20	1700	30	> 1700	15

Type C strains (*Cl. bubalorum*) grew poorly in the liquid media usually employed for toxin production; on two occasions γ-toxin appeared to be produced. This finding was confirmed by serum tests on the concentrated filtrates, but further subcultures of these strains failed to yield any toxin. The organism grew well only in meat broth with added meat particles; no toxin was produced in this medium, nor in any medium so far tried has any toxin but γ been demonstrated.

Antigenic unity of α-toxin of Cl. oedematiens

We had obtained no evidence that any of our toxins possessed any lethal activity, at any rate in the concentrations normally occurring, except α-toxin. A lethal toxin is produced by both types A and B strains; on laboratory media, however, type B strains produced much more than type A, and many of the type A strains did not produce enough to be detected. It remained therefore to prove or disprove the antigenic unity of the lethal toxin. Serum tests were therefore set up, using both types A and B antisera, against one filtrate derived from *Cl. oedematiens* type A and another derived from type B; after standing for 1 hr., 0·5 ml. of the filtrate-serum mixture was injected subcutaneously into mice. The end-point was taken as death of half the mice injected within 48 hr. Table 9 shows that within the limits of error of the test there was no difference between the anti-lethal values of the sera against the different filtrates; no evidence is therefore available that α-toxin is complex.

Table 9. *Comparison of anti-lethal values of sera against* Cl. oedematiens *type A and type B filtrates, to show antigenic unity of α-toxin*

	Anti-lethal values against			Anti-lethal values against	
Type A serum	Type A filtrate AE 208	Type B filtrate AE 793	Type B serum	Type A filtrate AE 208	Type B filtrate AE 793
R 7870	300	300	2705	1700	1550
850 A	1900	1550	1141	750	750
R 7907	1150	1000	2096	1000	1000
97	800	720	2209	1700	1900
R 7869	460	470	T 3	6500	6000
R 7903	850	780	T 61	1700	1450
9559	1500	1450	2184	1850	1650

Thus there is evidence that including hyaluronidase there are at least seven antigenic substances present in *Cl. oedematiens* filtrates. The characteristics of those on which we have worked and their distribution among the types are given in Table 10. It is clear that examination for production of β and γ is adequate for typing.

Table 10. *Activities and distribution among types of* Cl. oedematiens *toxins*

		Presence in *Cl. oedematiens*		
Activities of toxin	Designation	Type A	Type B	Type C
Lethal, necrotizing	α	+	+	−
Haemolytic, necrotizing lecithinase	β	−	+	−
Haemolytic lecithinase	γ	+	−	?+
Oxygen-labile haemolysin	δ	+	−	−
Opalescence in lecitho-vitellin; ? pearly layer	ϵ	+	−	−
Haemolysin	ζ	?−	+	−

Practical application to typing

For routine examination strains were grown in 10 ml. amounts of Brewer's medium (Brewer, 1940; Hayward, 1943) in test-tubes, the organisms removed by centrifugation or filtration through paper after mixing with a little kieselguhr or 'Hyflo-Supercel' and the filtrate tested as in Table 11.

The results should be read immediately after 1 hr. in the water-bath at 37° and after not less than 2 hr. at room temperature. β- and γ-toxins produce opalescence in L.V. in the water-bath, but are hot-cold haemolysins. If tubes 2 and 4 show no opalescence in the bath, but show a progressive opalescence starting from the bottom of the tube when they are cooled, ϵ is present. Few of our sera possessed sufficient ϵ-antitoxin to neutralize ϵ in these tests. If it is desired to avoid trouble with ϵ, dilution of filtrates 1 : 4 will usually prevent its action. δ and ζ produce rapid haemolysis in the water-bath. No difficulty has arisen in typing all the strains by this method.

A much more convenient way is to carry out typing on plates; the method is similar to that used in the Nagler plate test for *Cl. welchii* (Hayward, 1943). The plates are made with Weinberg's V.F. agar containing about 5 % of L.V. After drying for 3–4 hr., half the plate is spread with 0·1 ml. serum containing about 200 units/ml. of γ-antitoxin or about 20 units/ml. of β-antitoxin. The serum is rapidly absorbed by the agar and after about ½ hr. the plate can be inoculated. After 2 days' incubation in the anaerobic jar, the colonies are examined.

Table 11. *Arrangement for qualitative determination of toxins present in a* Cl. oedematiens *filtrate*

Tube	Contents		Indicator	Results					
1	1 ml. filtrate + 1 ml. S.A.B.		0·5 ml. L.V.	+	+	+	+	+	+
2	1 ml. filtrate + 20 units γ-A.T.			+	+	–	–	(+)	(+)
3	1 ml. filtrate + 10 units β-A.T.			–	–	+	+	+	+
4	1 ml. filtrate + $(20\gamma+10\beta)$ units A.T.			–	–	–	–	(+)	(+)
5	1 ml. filtrate + 1 ml. S.A.B.		0·5 ml. washed horse red cells	+	(+)	+	(+)	+	(+)
6	1 ml. filtrate + 1 ml. S.A.B.	+ $(7\delta+7\zeta)$ units A.T.		(+)	(+)	(+)	(+)	(+)	(+)
7	1 ml. filtrate + 20 units γ-A.T.			(+)	(+)	–	–	–	–
8	1 ml. filtrate + 10 units β-A.T.			–	–	(+)	(+)	(+)	(+)
9	1 ml. filtrate + $(20\gamma+10\beta)$ units A.T.			–	–	–	–	–	–
			Toxins present	$\beta+\zeta$	β	$\gamma+\delta$	γ	$\gamma+\delta+\epsilon$	$\gamma+\epsilon$

+ = Immediate reaction in water-bath.
(+) = Delayed reaction (haemolysis or opalescence) on cooling.
A.T. = antitoxin.

Results. All the type A strains produced γ-lecithinase; all the type B strains produced β-lecithinase; no strain produced both β and γ. The appearance of the colonies gave other useful information. Thus the colonies of type A strains on egg agar were covered with a pearly layer; the lecithinase reaction was well marked and tended to be arranged in concentric rings (Plate 1, fig. 1), whereas the type B colonies (Plate 1, fig. 2) were surrounded by a diffuse lecithinase reaction and showed no pearly layer. Of the forty doubtful strains resembling *Cl. oedematiens* more closely than any other described anaerobe, but differing from it in one or other characteristic, thirty-five produced no lecithinase under our conditions either in liquid culture or on plates, while five produced lecithinases antigenically distinct from both β and γ.

It is of course essential in attempting diagnosis in this way to use sera which do not contain appreciable amounts of antibody to lecithinases for which one is not testing (e.g. *Cl. welchii* α-toxin); if for instance the oedematiens type A serum used contains sufficient antibody to β-toxin, it will inhibit both β- and γ-lecithinase reactions; if it contains sufficient antibody to *Cl. welchii* α-toxin, it will inhibit the reaction due to this toxin also. The more information there is available about the serum values of the sera used, therefore, the more certainly can they be used for typing.

Comparison with other typing methods

Fermentation reactions. Scott *et al.* (1934) separated type A *Cl. oedematiens* from type B on the ground (among others) that the former fermented glycerol, while the latter did not. There is not much agreement in the literature on the sugars fermented by these organisms, though Turner (1930) in an exhaustive survey of the subject states that on the medium he recommended both types A and B ferment glucose and maltose, while type A in addition ferments glycerol. Keppie (1944) on the other hand (admittedly on a somewhat different medium) found that a few strains of type B fermented glycerol, though less vigorously than type A strains.

For our medium we have used: 1 % peptone-water with 0·5 % NaCl, 0·25 % agar and 0·5 % of the test sugar, with bromcresol-purple as indicator. The carbohydrates used were: glucose, maltose, lactose, sucrose, mannitol, salicin and glycerol. Anaerobic conditions essential for good growth of *Cl. oedematiens*, particularly of type B strains, are made possible by the addition of the agar, thus avoiding the use of the anaerobic jar. On this medium some anaerobes bleach the indicator without production of acid; all tubes showing colour change were therefore spot-tested with indicator. No strain certainly *Cl. oedematiens* produced bleaching, but as this activity is not uncommon among anaerobes and is characteristic of *Cl. sporogenes* and *Cl. histolyticum* for example, the test was a convenient screen for examining the unidentified strains, many of which fermented the same sugars as *Cl. oedematiens* but in addition bleached the indicator in all the other sugars tested. One essential must not be forgotten—the organism must grow well in the medium, or false negatives will be found, as was often the case with type B strains.

Table 12. *Fermentation reactions of fifty-nine strains of* Cl. oedematiens

	Carbohydrate tested							
Type	Glucose	Maltose	Lactose	Sucrose	Mannitol	Salicin	Glycerol	Number of strains
A	+	+	−	−	−	−	+	35*
	+	+	−	−	−	−	−	2
	+	−	−	−	−	−	+	3
	+	−	−	−	−	−	−	5
								Total 45
B	+	+	−	−	−	−	+	7
	+	+	−	−	−	−	−	2*
	+	−	−	−	−	−	+	3
	+	−	−	−	−	−	−	2
								Total 14†

+ = Fermentation with production of acid and gas. − = No fermentation.

* 'Typical' strains (criteria of Scott *et al.* 1934).

† The remaining two strains could not be obtained pure.

Table 12 shows the considerable variation among our strains; though each was consistent in its habits, the results we obtained by no means always agreed with those expected from typing. We are therefore disinclined to rely on fermentation reactions alone for identifying and classifying *Cl. oedematiens*.

Growth and colony form. Type A strains grow more profusely on plates than type B strains, the colonies are denser and slightly raised. Type B strains grow more slowly, require more exacting anaerobic conditions, and produce flatter more filamentous colonies. Differentiation by colony form is, however, very difficult.

Somatic and flagellar antigenic structure (Turner & Eales, 1943; Keppie, 1944). All *Cl. oedematiens* strains appear to have two somatic antigens in common; one of these is shared with *Cl. haemolyticum.* The thirty-three strains examined by Turner & Eales (1943) were separable (on the basis of their H-antigenic structure) into eleven groups, seven of which contained only one strain. Such antigenic analysis is of fundamental importance; but judging by Turner & Eales' tables such analysis cannot be used to separate *Cl. oedematiens* from other genera of *Clostridium*, nor the three types from one another, without very extensive investigation.

Table 13. *Lecithinase and pearly layer reactions of* Cl. oedematiens *and other* Clostridium *species*

		Inhibition of lecithinase reaction by sera containing		
Organism	Lecithinase reaction	*Cl. oedematiens* γ-antitoxin	*Cl. oedematiens* β-antitoxin	Pearly layer
Cl. oedematiens Type A	+	+	−	+
Type B	+	−	+	−
Cl. haemolyticum	+	−	−	−
Cl. welchii (all 4 types)	+	−	−	−
Cl. sporogenes	+	−	−	+
Cl. histolyticum	−	−	−	−
Cl. sphenoides	+	±	−	−
Cl. sordellii	+	(+)	(+)	+

Differentiation of Cl. oedematiens *from other lecithinase-producing anaerobes*

Several other species of *Clostridium* produce lecithinase or pearly layer reactions on plates (Table 13). *Cl. haemolyticum*, regarded by some (e.g. Weinberg, Nativelle & Prévot, 1937) as a member of the *Cl. novyi* (*Cl. oedematiens*) group, produces large amounts of a haemolytic lecithinase, not neutralized in our tube or plate tests by either *Cl. oedematiens* β- or γ-antitoxins, but readily neutralized by the single anti-haemolyticum serum available. The lecithinase and pearly layer reactions of the remainder present no difficulty as they are not inhibited either by β- or γ-antitoxin, but the reactions of *Cl. sordellii* are of some interest, for after 24 hr. incubation they appeared to be slightly inhibited by sera containing either β- or γ-antitoxin; no inhibition could be demonstrated at 48 hr. After 24 hr. as judged by our tests *Cl. sordellii* might perhaps have been classified as a feebly toxigenic strain of *Cl. oedematiens*; fortunately, its cultural and biochemical characters differ so greatly from those of *Cl. oedematiens* that confusion with it is improbable.

All the strains available were tested on these plates and gave clear-cut results. In addition it was found possible to identify *Cl. oedematiens* in impure culture, both by testing the filtrates and by the plate reactions, although if the cultures were heavily contaminated the plates and haemolytic tests were difficult to read.

It must be clearly understood, however, that the tube and plate tests here described provide only indications, however useful, that particular toxins are produced by the strains under test. Proof that a given filtrate contains, say β-toxin, requires the demonstration that in suitable tests the filtrate is neutralized by β-antitoxic sera in proportion to their β-antitoxin content

Table 14. *Comparison of anti-β and anti-haemolyticum-lecithinase values of sera in L.V. tests, to show that two* Cl. haemolyticum *filtrates contain* Cl. oedematiens *β-toxin*

Serum	Anti-β value against filtrate OC23445	L.V. value against *Cl. haemolyticum* filtrate OC21846	OC22846
2561	31	36	36
2025	100	100	125
2147	200	230	230
T61	500	500	500
850A	1600	1600	1550
T3	3250	2800	2800

Proof that a particular filtrate contains a toxin antigenically distinct from β-toxin, but having similar properties, requires a demonstration that the minimum effective dose of the filtrate against indicators affected by β-toxin is unaffected by the presence of β-antitoxin.

These points are well illustrated by *Cl. haemolyticum*. Two strains of *Clostridium*, identified by others as *Cl. haemolyticum* and having the cultural characters of that organism, produce a lecithinase not neutralized in our tube or plate tests by either β- or γ-antitoxin. This is due to the fact that the amount of lecithinase produced is far greater than the sera used can neutralize. When the filtrates are diluted considerably, serum-value tests with L.V. as indicator (Table 14) show that the filtrates contain β-toxin (cf. Sordelli & Ferrari, quoted by Weinberg *et al.* 1937). This finding supports the view that *Cl. haemolyticum* is a member of the *Cl. oedematiens* group; whether it is a member of type B or a separate type can only be decided when more strains are available for test.

DISCUSSION

When Scott *et al.* (1934) divided the 'oedematiens' group into three types, they based their division on source, size, fermentation of glycerol and pathogenicity. It is evident that though their criteria are not as stringent as was at first thought, the conclusions they drew from them are sound; our results support their views and provide type-differentiating criteria of greater stringency. Hayward & Gray (1946) divide *Cl. oedematiens* type A into typical and atypical strains on the basis of their fermentation reactions; by their haemolysin test ten out of eleven typical, and eight out of twenty atypical strains, were diagnosed as *Cl. oedematiens*. Putting the matter another way, one typical and twelve atypical strains failed to produce the specific haemolysin. Whether they regard this as a criticism of the haemolysin test is not clear; but if, as we believe, their haemolysin is our γ-toxin, we are not surprised that they failed to demonstrate it in some instances, as the haemolysin test is so much less sensitive than the L.V. test. They state: 'The atypical *Cl. oedematiens* includes strains that fermented lactose or sucrose failed to liquefy gelatine or failed to ferment maltose, but resembled *Cl. oedematiens* in all other morphological, cultural or biochemical characters.' Unfortunately, it is not clear which of the atypical strains gave a positive haemolysin test, or whether lactose or sucrose fermenters were ever diagnosed as *Cl. oedematiens* by its use. In our hands all our thirty-five typical type A strains and all ten atypical strains (atypical only in that they failed to ferment maltose or glycerol) produced γ-lecithinase. All the thirty-five strains of unclassified anaerobes that were regarded as distinct from *Cl. oedematiens* on other grounds (morphology, cultural characteristics) failed to produce γ-lecithinase; the lecithinases or haemolysins of the few unclassified strains that produced them were antigenically distinct from β-, γ-, δ- and ζ-toxins. Most of the unclassified anaerobes fermented glucose, maltose and glycerol; several fermented sucrose and lactose mannitol or salicin. Of our fourteen type B strains only two were typical (Table 12); all fourteen produced β-lecithinase. Evidently capacity to produce β- or γ-lecithinase is a far more consistent character than capacity to ferment a particular sugar.

We feel that *Cl. oedematiens* (all types) is best defined as that species of *Clostridium* possessing Somatic (O) antigens Oed. I or II or both. It is an obligate anaerobe, usually fermenting glucose and maltose and often fermenting glycerol and not fermenting lactose, sucrose, mannitol or salicin; it does not bleach bromcresol-purple. If this definition be accepted *Cl. haemolyticum* is included in *Cl. oedematiens*, but determination of its exact position requires the examination of more strains.

The species so defined is readily divisible into three types based on toxin production, type A producing γ-toxin, type B β, and type C none. The types thus differentiated show marked internal consistency and agree very well with those based on source, size, cultural characters and pathogenicity. Types based on fermentation reactions or flagellar antigenic analysis alone show no such internal consistency, cut sharply across classifications based on other characters, and are too numerous to be of practical use.

Finally, we would draw attention to the fact that although β- and γ-toxins are lecithinases having the same type of enzymatic activity as the α-toxin of *Cl. welchii* (with which they also agree in being activated by calcium ions) they do not attack the same red blood cells as the latter toxin. *Cl. oedematiens* β- and γ-toxins readily attack horse red cells, which are hardly affected by *Cl. welchii* α-toxin; sheep red cells, which are readily attacked by *Cl. welchii* α-toxin, are relatively insensitive to *Cl. oedematiens* β and γ. Evidently something is involved in the haemolysis of these different types of red cell beside the enzymatic attack on lecithin.

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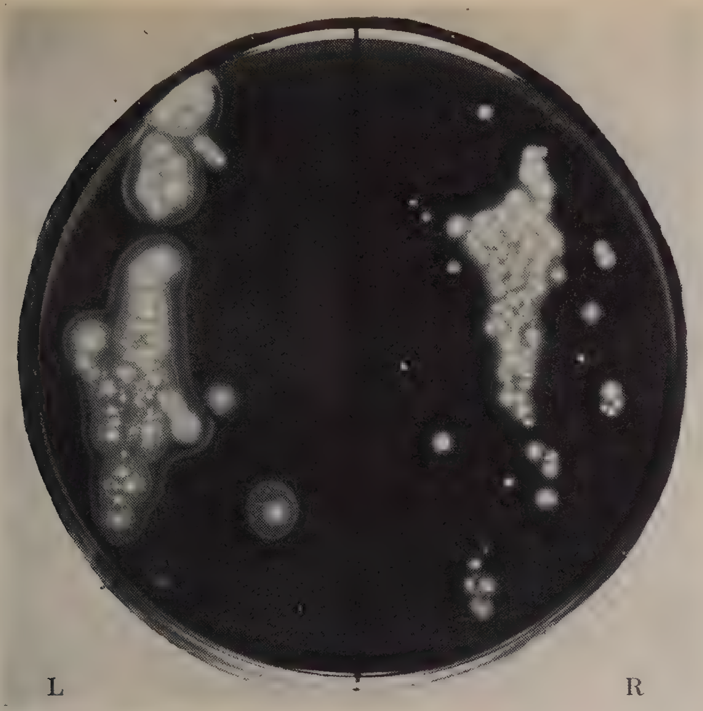

Fig. 1

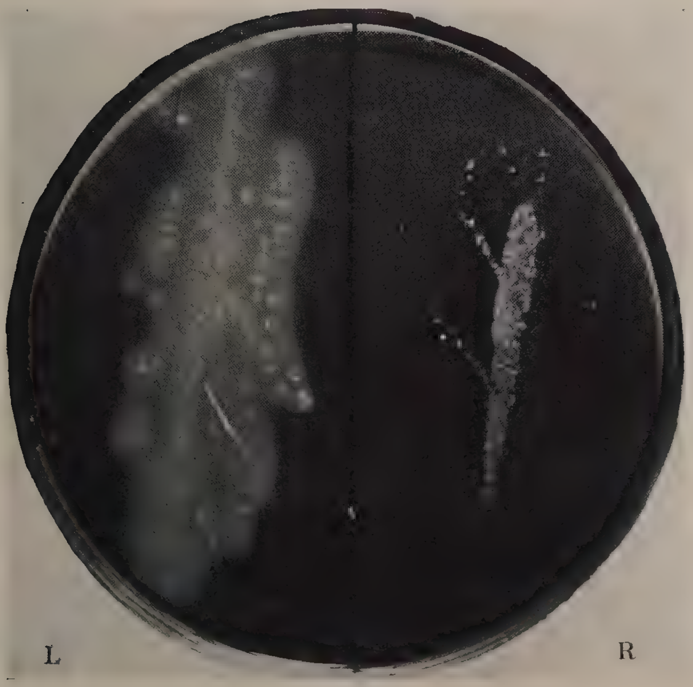

Fig. 2

C. L. Oakley, G. H. Warrack and P. H. Clarke—The toxins of *Clostridium oedematiens*. Plate 1

EXPLANATION OF PLATE

Fig. 1. *Cl. oedematiens* type A, strain no. 45. Anti-γ serum R7903. Left: culture, no serum. Concentric rings of lecithinase reaction (γ-toxin). Right: culture + anti-γ serum. Inhibition of lecithinase reaction. Slight haloes due to pearly layer formation (? ϵ-toxin) which is not inhibited.

Fig. 2. *Cl. oedematiens* type B, strain 'Albiston'. Anti-β serum Ex949. Left: culture, no serum. Diffuse lecithinase reaction (β-toxin). No concentric rings or pearly layer formation. Right: culture + anti-β serum. Inhibition of lecithinase reaction.

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Bacterium melaninogenicum—A Misnomer

By HERTA SCHWABACHER and D. R. LUCAS
E.M.S. Sector IV Central Laboratory, Watford

and C. RIMINGTON
Department of Chemical Pathology, University College Hospital Medical School, London

SUMMARY: The morphological and cultural characteristics of seven recently isolated strains of *Bacterium melaninogenicum* closely resembled those given by previous authors. Fermentation reactions were unsatisfactory, owing to the production of acid in basal media without added carbohydrate. The growth of two strains studied in detail was greatly facilitated by X factor and to a much smaller extent by V factor; it was best on chocolate agar. The strains were non-pathogenic in mice. Serologically, they behaved alike in precipitin and complement-fixation tests. There was insufficient information to classify them into subtypes.

The black pigment, extracted by a new technique which avoids the use at any stage of alkalis, proved to be haematin (not melanin), united in the cells with a bacterial protein to form a parahaematin. Crystalline pyridine haemochromogen and haemin have been prepared from the isolated pigment. The haemin when coupled with renatured ox globin gave reconstituted methaemoglobin, oxy- and carboxy-haemoglobins. Ultra-violet and visible spectral absorption data support the identification of the pigments. In particular, the possible reduction of the vinyl groups of the porphyrin nucleus by the organism, leading to meso- or deuterohaematin, has been excluded.

It is suggested that the organism be assigned to the genus *Fusiformis*, and the specific name be altered from *melaninogenicus* to *nigrescens*, making *Fusiformis nigrescens.*

In 1921 Oliver & Wherry isolated an anaerobic Gram-negative cocco-bacillus from the human throat, urine and faeces, and from an infected surgical wound. The organism grew on human blood agar slopes after 1–2 weeks' incubation, using up haemoglobin during growth and giving rise to a confluent, jet-black growth. Believing the pigment to be melanin, they named the organism *Bacterium melaninogenicum.* Since then several authors have mentioned the organism, and some have studied it in detail.

This paper records the properties of seven recently isolated strains of *Bact. melaninogenicum* and the identification of the characteristic brown-black pigment as haematin, not melanin.

The strains were as follows: J. S. from pyorrhœa alveolaris, associated with micrococci and non-haemolytic streptococci; no. 8680 from an abscess of the jaw, associated with an *Actinomyces bovis*; no. 3999 and no. 4814 from the throat in Vincent's angina; no. 1136 from an abscess of the neck, associated with an anaerobic streptococcus and aerobic and anaerobic Gram-negative bacilli; no. 1362 from an abscess of the nasal septum which also yielded a *Staphylococcus aureus* and a diphtheroid; 'M' from a case of 'black tongue', associated with anaerobic Gram-negative bacilli and anaerobic streptococci.

DESCRIPTION OF THE STRAINS

Morphology

Microscopically the organisms showed no remarkable variation either among the different strains or when the same strain was grown on solid or in fluid media. The cells were mostly ovoid, measuring $0{\cdot}4$–$1{\cdot}0 \times 0{\cdot}3$–$0{\cdot}4\mu$. Occasional cells up to $8{\cdot}0\mu$. long were encountered. Some cells showed bipolar staining; no capsules could be demonstrated. The organisms were non-motile, non-acid-fast and did not form spores.

Cultural characters

Two per cent horse blood agar plate. Five days at 37°: smooth, shiny, flat-convex, jet-black colonies, about 1 mm. in diameter, with an entire edge. Haemolysis was first evident, after 2–3 days, in the heavily inoculated part of the plate, and the plates were practically cleared of haemoglobin after 14 days (Pl. 1, fig. 1). The colonies were brown at first.

Gelatin enriched with Fildes's extract and 2% peptone (Evans). Four weeks at 37°: a semi-opaque, mucinous mass of growth with liquefaction of the gelatin.

Fluid media. Growth did not readily occur in simple broth or peptone water. In Fildes's broth (Fildes, 1920) a ropy, mucinous deposit was produced in 2–3 days.

Loeffler's serum slope. Two days at 37°: confluent, colourless, moist growth.

Metabolism. All the strains were strictly anaerobic, and required the presence of about 5% CO_2 in the gas phase. All cultures had a foul odour.

The effect on growth of various media was measured by the average size of ten discrete colonies attained under comparable conditions by the strains 1136 and 4814. On nutrient agar, strain 1136 grew only in minute colonies and strain 4814 failed to grow. When 2% Evans peptone (Evans' Medical Supplies, Ltd., London) was added to the nutrient agar, both strains gave small colonies. The largest colonies were obtained on chocolate agar, the mean diameters for the two strains were 1·80 and 2·45 mm. compared with 0·14 and 0·45 mm. on blood-saline agar.

The effect of incorporating X and V factors (Thjøtta, 1921), supplied as yeast extract and haemin respectively, in nutrient agar, was as follows:

Strain	Agar with V (mm.)	Agar with X (mm.)	Agar with X and V (mm.)
1136	0·23	2·13	2·37
4814	0·61	1·48	2·42

Since strain no. 4814 failed to grow on nutrient agar and the colonies of both strains were largest when both X and V factors were present, it is reasonable to assume that both factors facilitated growth.

In confirmation of Slanetz & Rettger's (1933) results with fusiform organisms, potato extract stimulated the growth of strain no. 4814. Cysteine, which they also recommended, had some beneficial effect. As might be expected from the foregoing, all strains grew readily in Fildes's broth. A fall in pH took place in all fluids in which there was growth.

Biochemical. Fermentation reactions were impracticable because acid was produced in the basal media commonly used for sugar reactions. Six strains, incubated for a week in peptone (Evans) water, were indole-positive. In Fildes's agar stabs, six strains produced hydrogen sulphide, as judged by the blackening of lead acetate contained in a layer of agar on top of the stab.

Sensitivity to sulphonamides was tested on a series of nutrient agar laked-horse-blood slopes (Harper & Cawston, 1945) containing 220 mg./100 ml. of sulphanilamide, 8·5 mg./100 ml. of sulphapyridine, 18 mg./100 ml. of sulphathiazole, 4 mg./100 ml. of sulphadiazine, and less than 30 mg./100 ml. of sulphamethazine. The concentration of the sulphonamides was in each case a half-saturated solution at room temperature. At these concentrations, growth was inhibited only by sulphanilamide.

The minimal bactericidal concentration of penicillin was estimated in Fildes's broth; for five strains it was 0·3 unit/ml. and for the sixth 0·6 unit/ml.

Serology

Shevky, Kohl & Marshall (1934) produced rabbit antisera with titres of up to 1:10,240; clumping was granular rather than flocculent, and their strains were serologically homogeneous. Weiss (1937), following the technique of Heidelberger & Kendall (1931), extracted from two strains bacterial proteins which were antigenic. The proteins from these two strains were immunologically distinct by precipitin reaction. Cross-reactions occurred with group A *Strep. haemolyticus* antiserum. Weiss related his findings to those of Heidelberger & Kendall (1936) who used haemolytic streptococci, and suggested the existence of a conjugated carbohydrate protein, analogous to the artificial pneumococcus type III polysaccharide protein synthesized by Avery & Goebel (1931).

Preparation of anti-sera. A rabbit anti-serum was prepared by intravenous inoculation with growth washed off a blood-agar plate and killed in 0·25% formol saline. After 6 weeks its agglutination titre was 1:320. Since the granular nature of the suspensions made direct agglutination tests difficult to interpret, the precipitin and complement-fixation reactions were used.

Precipitin tests. The precipitin tests were set up by extracting growth from blood plates in 0·1 N-HCl for 10 min. at 100°, neutralizing with 0·5 N-NaOH, centrifuging and layering the supernatant fluid on to neat serum in the stem of a Pasteur pipette. An opaque ring was clearly visible at the interface of the two liquids within 10 min. All our strains reacted with the serum. No strain reacted positively with four sera prepared against strains of non-pigmented Gram-negative anaerobes of the *Fusiformis* type, isolated from human material during the last year; nor with Lancefield's A, B, C and G grouping sera (cf. Weiss, 1937).

Saline extracts, prepared by boiling a suspension for 5 min. or holding it at 37° for 2 hr., also gave a positive precipitin test. Intravenous inoculation into a rabbit of the saline extract, which by chemical test contained carbohydrate but not protein, failed to produce any antigenic response after 6 weeks.

Complement-fixation tests. The technique described by Price (1933) for the preparation of gonococcal antigen was used to prepare extracts for complement-fixation. Using the homologous antigen at 1:30 the anti-serum fixed complement to a titre of 1:480. When the test was carried out simultaneously on all seven strains, using each antigen at 1:30, titres of 1:160 in four cases and 1:320 in three resulted.

In an attempt to relate the precipitinogen to the complement-fixing antigen, a suspension of the homologous strain was divided into three portions. From the first portion antigen was prepared as above. The second and third portions were extracted in normal saline for 10 min. at 100° and 2 hr. at 37°, respectively, centrifuged and precipitinogen demonstrated in the supernatant fluid. The supernatant fluid from the second extraction contained no demonstrable precipitinogen. The remaining deposits were then extracted by Price's technique. The extract from the first portion fixed complement at a serum titre of 1:320. The final extracts from the second and third portions partly fixed complement in the 1:20 dilution, thus showing that, when precipitinogen was removed from the bacterial cells, the latter were incapable of fixing complement in the usual way. It is therefore probable that the two serological activities, precipitation and complement-fixation, are referable to the same antigen. This is substantiated by the fact that saline extract fixed complement to titre.

Animal pathogenicity

Burdon (1932) described one strain which after subcutaneous inoculation into rabbits and guinea-pigs produced extensive cutaneous gangrene and death after 48 hr.; Weiss (1943) described similar severe effects in rabbits with recently isolated strains. Apart from these reports, claims for pathogenicity are somewhat unconvincing or based on indirect evidence.

Four tests were made of mouse pathogenicity. In the first intravenous, intraperitoneal and intramuscular injections were tried. In the second a recently isolated strain was inoculated intraperitoneally. In the third and fourth, another recently isolated strain was inoculated intraperitoneally, suspended in 2·5 and 5·0% 'biomucine' (Laboratoires Robert et Carrière). The results were uniformly negative.

Comparison with previously described strains

Reference to the literature shows that the organism has been recovered chiefly from the mouth, genitalia and sites of chronic suppuration. It is a strict anaerobe, grows slowly, and is difficult to separate from contaminants. Cultures have a foul smell. Morphologically it is Gram-negative, non-motile, non-sporing and non-acid-fast. Shevky *et al.* (1934) and Weiss (1943) claim to have seen a capsule in clinical material and in smears from animal autopsy respectively, but other authors do not describe capsule formation. There is slight variation in the descriptions of the bacterial cell, but all agree that it is polymorphous and that bacillary and coccal forms coexist. Shevky *et al.* (1934) mention bipolar staining in the coccal forms.

The black pigment is formed slowly, and its formation is accompanied by the disappearance of haemoglobin from the medium. No pigment is formed in the absence of blood; Shevky *et al.* (1934) say that on serum agar there is late production of a brownish pigment.

These observations are in substantial agreement with those made in this paper. On the biochemical reactions of the organism there is wide disagreement, especially with regard to fermentation reactions. There is considerable variation of opinion on the capacity of this organism to liquefy gelatin; most authors give the indole reaction as positive.

There is, nevertheless, sufficient correspondence to leave no doubt that the organisms described in this paper are identical with those previously described as *Bact. melaninogenicum.*

THE PIGMENT

Hitherto it has been generally accepted that the black pigment produced by the organism is melanin or a melanin-like pigment. Considering that the pigment is formed only in the presence of blood, that its formation coincides with the disappearance of haemoglobin and that neither adrenalin nor ascitic fluid assist in its formation (Shevky *et al.* 1934), it seemed more likely to be a breakdown product of haemoglobin.

The evidence upon which the pigment was first described as a melanin by Oliver & Wherry (1921) is entirely inadequate. They noted its insolubility in several organic solvents and recorded that it occurred as extracellular amorphous masses; it was also found to dissolve slowly in sodium hydroxide solution, but apparently no spectroscopic investigations were made. Shevky *et al.* (1934) confirmed the insolubility of the pigment in solvents such as acetone, chloroform, etc., and its solution in weak alkalis, again without recording any spectroscopic examination.

Our own observations have shown that the pigment is readily dissolved from the bacterial growth by pyridine, in which it forms a greenish brown solution with maximum spectral absorption in the region of 533mμ. Addition of a small quantity of sodium hydrosulphite ($Na_2S_2O_4$) produced a rapid change in colour to brownish red, and at the same time the intense and characteristic absorption bands of pyridine haemochromogen (pyridine ferroprotoporphyrin) appeared (maxima 557·4 and 528·3 mμ.). This behaviour is consistent with that of a haem pigment, but in view of the fact that the organisms had been removed from an agar plate originally containing blood, rigorous purification to remove all haemoglobin derived from the medium was essential before such a test could be considered unequivocal. It was, in addition, highly desirable that crystalline derivatives should be prepared in order to support any spectroscopic evidence. It is, however, well known that treatment with alkalis such as sodium hydroxide, the only good and easily manipulated solvent for haematin, so alters this pigment that it is frequently impossible to prepare crystalline derivatives from it after such extraction (Fischer, 1924; Keilin, 1943). Moreover, solution of haematin in sodium hydroxide and reprecipitation at the isoelectric point failed to eliminate accompanying protein, which largely reprecipitated with the pigment. A new extraction and purification technique was required which would avoid the use at any stage of sodium hydroxide. Such a technique was found in the use of 90% phenol as described below.

Extraction and purification of the pigment

Plates were used that showed a good growth of organisms and from which the haemoglobin had almost entirely disappeared. The growth was removed with as little adhering agar as possible, stirred up with water at 50–60° and centrifuged, the supernatant being discarded. Washing in this manner was repeated until all traces of agar and haemoglobin were removed. The pigmented organisms were then stirred with about 20 times their volume of 90% phenol (10 ml. of water + 90 g. phenol). Extraction of the colouring matter proceeded slowly, and it was found convenient to remove each lot of phenol solution by centrifugation after 2–4 hr. contact (first extraction 12 hr.) with the bacterial mass. When the solvent no longer extracted spectroscopically detectable pigment (i.e. a well-defined broad band with centre 627·5 mμ.) from the cell debris, the extracts were combined, and to the dark brown solution twice its volume of absolute ethanol was added. This served to precipitate a small quantity of dissolved protein which was removed by a no. 4 sintered glass filter.

The solution was then dialyzed in a collodion bag (Visking) against tap water. Separation of two liquid phases soon occurred, and it was necessary to mix the contents of the bag frequently. Finally, when all but a trace of phenol was removed, the dark pigmented bottom layer showed signs of turbidity due to precipitating pigment. It was found convenient to remove it at this stage into a centrifuge tube, add plenty of distilled water, stir, centrifuge down the granular pigment and repeat the water washing two or three times.

We believe that by this process we obtained the pigment with as little chemical alteration as possible. That it no longer contained or was associated with protein was demonstrated by the fact that a solution in dilute NaOH, when reduced by $Na_2S_2O_4$, gave only the bands of reduced haem and no trace of a haemochromogen. Dissolution in alkali was, however, rather slow, a phenomenon frequently encountered with preparations of haematin isolated from natural sources.

Preparation of crystalline derivatives

When a small portion of the isolated pigment was mixed on a glass slide with a drop of Takayama's reagent (Harrison, 1944) it dissolved. A cover-slip was applied, and after a few minutes the colour changed to the red of pyridine haemochromogen and crystal masses were visible under the microscope. Even when prepared from pure haematin the shape of these crystals was variable, ranging from prismatic needles grouped in stellate clusters to flat overlapping plate-like structures. We observed that the type of crystal produced depended somewhat upon the maturity of the Takayama reagent and hence on the velocity of reduction, and also that the needles first formed tended to become transformed on the slide into plates. Control experiments were therefore always done at the same time with pure crystalline haemin. Pl. 1, figs. 2–7, record some of the preparations obtained.

The haemin (Pl. 1, fig. 8) was similarly obtained from the bacterial pigment by warming on a slide with acetic acid-KCl mixture, as in preparing Teichmann's crystals from blood.

Properties and nature of the pigment

That the pigment isolated by the phenol method was a haem pigment was readily demonstrated by the spectroscopic examination of its solutions in different solvents, by the formation of a pyridine haemochromogen and, lastly, of a porphyrin when the pigment was treated with concentrated sulphuric acid. These results are presented in Table 1, where the data for haemin similarly treated are given for comparison. The specimen of haemin was a sample recrystallized as described by Rimington (1942), and all measurements were made with the Hartridge reversion spectroscope. It will be seen from the spectroscopic data that in all respects the bacterial pigment very closely resembled haematin (ferriprotoporphyrin). The possibility, however, had to be borne in mind that a reduction of the two vinyl groups $-CH{=}CH_2$ of the protoporphyrin nucleus might have been brought about by the bacterial cell, leading to mesohaematin, the Fe complex of mesoporphyrin, or even that the vinyl side chains might have been completely removed, resulting in deuterohaematin, the Fe complex of deuteroporphyrin. Both these porphyrins have been found in faeces, where they are undoubtedly derived from protoporphyrin by bacterial action (Zeile & Rau, 1937).

The close correspondence of the absorption spectra of the pigment of *Bact. melaninogenicum* with haematin, and of the porphyrin derived from it with haematoporphyrin rather than mesoporphyrin or deuteroporphyrin (see Table 1), strongly suggests that the vinyl groups remain intact. Drabkin (1942) has shown that the presence of free vinyl groups in haem pigments displaces the absorption maxima about 11 mμ. towards the red, the α band of pyridine haemochromogen, for example, lying at 558 mμ. and that of pyridine mesohaemochromogen at 547 mμ. Further evidence was obtained, however, by coupling the bacterial pigment with native globin to form methaemoglobin, reducing this to oxyhaemoglobin with Stokes's reagent, and measuring the absorption bands of these pigments and the CO haemoglobin obtainable by saturating the solution of the oxy compound with coal gas. Measurements were also made of the Soret bands in the near ultra-violet, and a complete set of data was simultaneously obtained upon an authentic specimen of reconstituted haemoglobin for which the starting point was pure crystalline haemin.

Preparation of reconstituted haemoglobins

Globin hydrochloride was prepared from ox blood (ox globin being relatively more stable than horse or human globin) and partially renatured by dialysis for 24 hr. against running tap water according to the method of Anson & Mirsky (1929–30). Dialysis was completed against M/30-K_2HPO_4 solution at the pH of which denatured globin is insoluble. After centrifugation the supernatant liquid, containing the renatured protein, was carefully adjusted by KH_2PO_4 solution to a pH of about 6·0. To separate portions of this solution were added solutions of bacterial pigment or crystalline haemin, respectively, in dilute NaOH, until the band of methaemoglobin at about 630 mμ. was clearly visible. After this measurement, freshly prepared Stokes's reagent (Harrison, 1944) was added drop by drop until the colour changed from brown to red and the methaemoglobin spectrum had given place to that of oxy-

Table 1. *Spectroscopic data obtained from the bacterial pigment and from crystalline haemin*

Solvent, treatment, etc.	Pigment from *Bact. melaninogenicum* (wave-length, mμ.)	Haemin (wave-length, mμ.)	Remarks
0·1 N-NaOH extract of growth	613·2	—	—
Do. + $Na_2S_2O_4$	558·3	—	Haemochromogen type spectrum
	527·9	—	
Pyridine extract of growth	532·8	—	—
0·1 N-NaOH solution of isolated pigment	613·2	615·7	Broad band
Do. + $Na_2S_2O_4$	573·8	575·0	Broad band
	535·5	540·0	Broad band
0·1 N-NaOH solution + pyridine + $Na_2S_2O_4$	557·2	557·2	Very sharp
	527·9	529·8	—
90 % phenol	627·1	627·6	—
	540·3	540·0	—
Acetic acid	629·1	633·1	Broad band
	538·2	541·4	Broad band
Ether-HCl	637·6	637·6	—
	543·6	543·7	—
Conc. H_2SO_4	601·9	601·7	Very sharp
	557·2	556·6	—
Porphyrin from above in pyridine	625·6	625·8	Very sharp
	579·6	579·3	—
	540·0	540·3	—
	507·5	507·8	—
Haematoporphyrin in H_2SO_4	—	601·6	Very sharp
	—	554·3	—
Mesoporphyrin in H_2SO_4	—	594·5	—
	—	551·9	—

haemoglobin. Over-reduction led to the single, broad band of reduced haemoglobin (purple-coloured solution), but shaking with air restored the two-banded HbO_2 spectrum. This demonstrated incidentally the capacity of the synthetic globin-pigment complex made from the bacterial pigment to function, like haemoglobin, as an oxygen carrier.

It was observed that during the addition of the Stokes's reagent some unavoidable denaturation occurred, so the solutions were centrifuged before proceeding further from this stage. For the measurement of the Soret bands, the reconstituted haemoglobins were equilibrated to pH 7·2 by dialysis against M/30-phosphate buffer, since the exact position of this maximum is influenced by the pH of the solution. Good agreement between the reconstituted bacterial pigment and that from haemin was again found for both the oxy and carboxy derivatives (Table 2). The instrument used was a Hilger medium quartz spectrograph, and the intensity of the Soret bands was found to be of the order of 10 times that of the visible bands, as is the case with normal native oxyhaemoglobin. The actual maxima of the Soret bands were in each case at a wave-length shorter than that usually observed in the case of native HbO_2 and HbCO: 405·5 mμ. as compared with the usual 414·5 mμ. for HbO_2 and 416·3 mμ. as compared with the usual 420·0 mμ. for HbCO.

Table 2. *Spectroscopic data from reconstituted haem-ox globin pigments*

Pigment type	**Bacterial pigment-ox globin (wave-length, mμ.)**	**Haematin-ox globin (wave-length, mμ.)**
Met Hb (alk.)	**596·7**	**—**
	538·9	**—**
Met Hb (acid)	**631·4**	**631·8**
HbO_2	**577·7**	**577·7**
	539·6	**539·4**
HbCO	**574·5**	**572·1**
	537·2	**537·8**
Pyridine haemochromogen	**557·1**	**557·2**
	528·3	**529·8**
	Soret bands	
HbO_2	**405·5**	**405·8**
HbCO	**416·3**	**416·0**

These shorter wave-lengths are those observed for the Soret bands on solutions of normal native HbO_2 and HbCO respectively which have been kept for some time and have apparently undergone some preliminary form of denaturation, which does not, however, appear to affect the visible band wave-lengths. In the case of the reconstituted bacterial pigments, the visible bands agreed closely with those of normal native HbO_2 and HbCO. The wave-lengths observed for the Soret band in these reduced reconstituted pigments and in partially denatured haemoglobins almost coincided with that of methaemoglobin. The presence of a Soret band is in itself strong evidence for the existence of the intact porphyrin ring.

State of the pigment in the bacterial growth

From all the evidence assembled above there remains no reasonable doubt that the pigment extracted from a culture of the organisms is identical with haematin (ferriprotoporphyrin). The next question to be considered was whether it was present in the bacterial mass as such or combined with bacterial protein; extractants such as pyridine and phenol might easily free haematin from a weak chemical union. The fact that solutions of the purified pigments in dilute NaOH gave no haemochromogen on reduction but that a simple extract made by stirring the growth with the same solvent afforded an intense haemochromogen spectrum on addition of $Na_2S_2O_4$, leads us to believe that in the cultures the haematin is united to a protein of the bacterial protoplasm, forming a parahaematin. This, indeed, might be anticipated from the known ease with which labile compounds are formed between proteins and the haem pigments.

DISCUSSION

The medical importance of *Bacterium melaninogenicum* is difficult to assess, and the observations in this paper have no direct bearing on it. The frequent association of the organism with pyorrhoea alveolaris, as noted by Liebetruth (1934) and Burdon (1928), is perhaps worthy of mention. By breaking down extravasated blood the organism might well be responsible for the black flakes which form in the gum pockets of pyorrhoea.

Failure to isolate the organism in routine cultural work is no doubt related to its slow growth and carbon dioxide requirements. The carbon dioxide requirement is the probable explanation of the difficulty previous workers have found in maintaining the organism in pure culture. The biochemical reactions reported in the literature are not sufficiently reliable to attempt classification into biochemical types, and there is insufficient information on serological work to deny or affirm the existence of serological types.

The organism is classified by Wilson & Miles (1945) in the group *Fusiformis*, and its morphological and cultural characteristics are quite in accordance with their general definition of the group.

The name *melaninogenicum* is a misnomer, indicating a biochemical activity of which the organism is incapable. By analogy the name should now be *haematinogenicum*; this, however, is long and ugly, and we suggest in place of the innaccurate *melaninogenicum*, the species name *nigrescens*, indicating accurately the progressive production of a black pigment.

We are indebted to Mr W. J. Fincham for his assistance in the preparation of the special media, to Mr E. M. Jope for kindly recording the ultra-violet absorption spectra of the reconstituted haemoglobin derivatives, and to Miss H. Sheinman who assisted with the chemical work on the pigment. The Laboratoires Robert et Carrière kindly supplied the biomucine. One of us (C.R.) acknowledges a grant from the Central Research Fund of the University of London, out of which the Hartridge reversion spectroscope was purchased.

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EXPLANATION OF PLATE

Fig. 1. 6-day-old culture of *Bact. melaninogenicum* showing clearing of medium by the organism; original size.

Fig. 2. Pyridine haemochromogen from pigment of *Bact. melaninogenicum*. Prismatic needle forms with some plates in aggregates; × 760.

Fig. 3. Pyridine haemochromogen from pigment of *Bact. melaninogenicum*. Transition into flat plates; × 570.

Fig. 4. Pyridine haemochromogen from pigment of *Bact. melaninogenicum*. Overlapping thin plates, frequently with upturned edges; × 760.

Fig. 5. Pyridine haemochromogen from haemin. Needle forms in aggregates; × 200.

Fig. 6. Pyridine haemochromogen from haemin. Overlapping thin plates (note upturned edges); × 870.

Fig. 7. Pyridine haemochromogen from haemin. Final form; × 300.

Fig. 8. Haemin from pigment of *Bact. melaninogenicum*; × 570.

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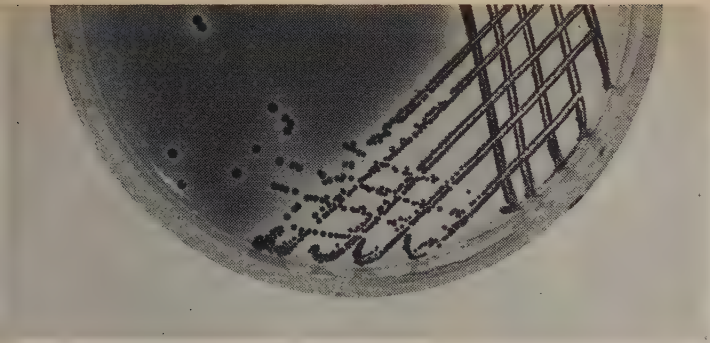

Fig. 1

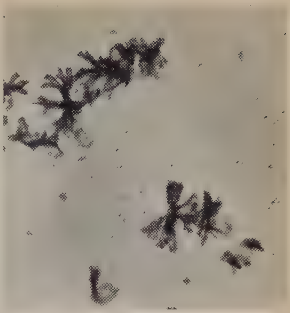

Fig. 2

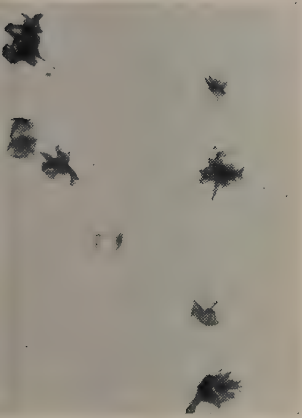

Fig. 3

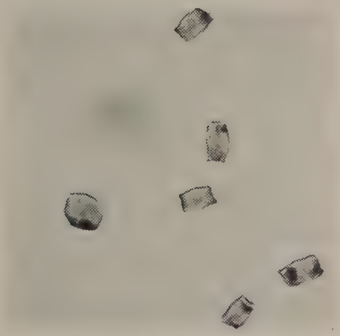

Fig. 4

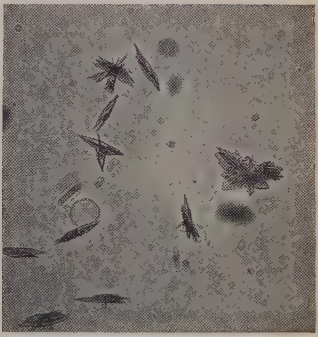

Fig. 5

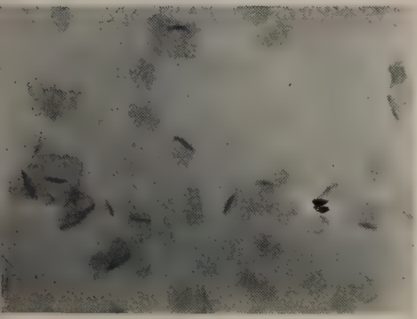

Fig. 6

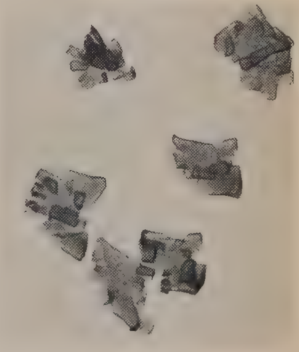

Fig. 7

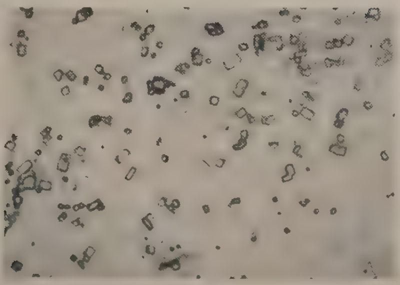

Fig. 8

H. SCHWABACHER, D. R. LUCAS AND C. RIMINGTON—*BACTERIUM MELANINOGENICUM*—A MISNOMER. PLATE 1

The Effect of Temperature on the Growth of *Bacterium coli* at pH 7·0 with a Constant Food Supply

By R. C. JORDAN and S. E. JACOBS

From the Physiology Institute, University College of South Wales and Monmouthshire, Cardiff, and the Bacteriological Laboratory, Imperial College of Science and Technology, London

SUMMARY: The effect of temperature on the growth of *Bact. coli* under accurately controlled conditions has been examined in an apparatus which permits food to be supplied at any desired rate by means of an automatic syringe mechanism. Six temperatures were used, from 15° to 40° at 5° intervals, with a single rate of food supply. Total and viable cell counts were frequently made and growth curves constructed. In some cases the changes in numbers were followed after stopping the food supply.

At all stages the total counts greatly exceeded the viable. There was always first an 'initial' phase in which the daily increments in total cells varied with time. Very early in this phase cell division lagged behind cell growth. At 15° this condition probably persisted throughout the whole experiment. The initial phase was longest when the temperature was lowest, but the time taken to reach 300×10^6 total cells/ml. did not vary greatly, except at 15° when the time was markedly increased. The yield of total cells at the end of the initial phase was highest when the temperature was lowest and depended on the amount of food added, i.e. on the duration of the phase, but relatively more cells were formed in a long than in a short phase. The viable cell count at the end of the initial phase was also enhanced by low temperature, except at 15° where the viable cells were fewer than expected in comparison with the other experiments. The ratio of the total to the viable count at the end of the initial phase was often close to 2·0.

The initial phase was followed by a 'steady' phase in which there were, on the whole, constant daily increments in both total and viable counts, although there were indications of stepwise increments. In the steady phase the calculated rates of increase in both total and viable cells were highest when the temperature was low, and bore a linear relation to the temperature. The rate of increase in viable cells was almost zero at 35°, which was apparently a critical temperature for viability. At 40° there was only a slow decline in viable cells, but nevertheless at both 35° and 40° growth of the cultures occurred since the total counts continually increased, but approximately half the cells formed were non-viable.

To express the difference between the total and viable counts a non-viability index was calculated. This was constant throughout the steady phase and was smallest when the temperature was lowest. The amount of food previously shown to be required for the formation of a new cell at 35° was found to be not inconsistent with the experimental data at any of the other temperatures, but if this amount were constant the food used per cell for maintenance and wastage must have declined with decreasing temperature.

After cessation of the food supply the total count declined at both 15° and 30°, being slightly faster at the lower temperature. The viable count also declined during starvation at 15°; the rate of decrease of the viable count was greater at 30°.

In a previous paper (Jordan & Jacobs, 1944) an apparatus for the cultivation of bacteria with a constant food supply was described, and the results of preliminary experiments with *Bact. coli* reported. It was shown that the apparatus would function satisfactorily and continuously for long periods, and that reproducible results could readily be obtained. These preliminary experiments were all conducted at 35° and pH 7·0, and resulted in the establishment of cultures in which the number of viable cells remained approximately constant while the total cells steadily increased; the numerical values attained depended on the rate of addition of food. The existence of these conditions enabled the calculation to be made of the amount of Difco dehydrated broth required for the formation of a new *Bact. coli* cell and the maintenance of its full activity apart from reproduction. Further experiments have now been performed with the same organism, apparatus and technique, in order to determine the effect of different temperatures at pH 7·0.

APPARATUS AND TECHNIQUE

Since the apparatus and technique were fully described previously (Jordan & Jacobs, 1944), full experimental details are not repeated here. A brief summary, however, may be given in order to make the present paper more self-contained. It was desired to study the effect of temperature on the changes in numbers of total and viable cells with time in cultures of *Bact. coli* which, instead of containing as is usual a large excess of food material, were supplied with food at a regular known rate.

Method of supplying the food. The food addition was effected by means of the automatic syringe mechanism described by Sims & Jordan (1942) which permits a known volume of sterile nutrient solution to be transferred to the culture at each operation of the syringe. The rate of addition was fixed at 0·066 ml. every 100 sec. using a solution of Difco dehydrated broth (6 g./l. before autoclaving), equivalent to an actual addition of 15·2 mg. of the dehydrated broth/hr.

The culture flask. This was a 5 l. Pyrex round-bottom flask with a central wide neck, around which were arranged three narrower necks. All were fitted with ground sockets, and the connecting fittings were provided with sealed-on glass dust-covers. The central neck served to admit the food solution and an air stream. The other three necks were for the effluent gases, for taking samples for counting purposes and to accommodate a modified dipstick for observing the level of the culture fluid. The final culture volume after autoclaving was 1450 ml. The connexions to the food supply were entirely of glass, no rubber being in contact with the food solution. The necessary flexibility in the glass connectors was obtained by spiralling the tubing.

The functions of the air stream. The culture was kept continuously stirred by means of a stream of dry sterile air warmed to the temperature at which the culture was to be grown. This air stream served a number of other purposes. By arranging the apparatus so that the jet through which the food solution entered the culture flask was surrounded by a glass guard tube which also served to admit the stream of air, contamination of the entering food by spray from the culture was prevented. The success of the technique largely depended on this.

The air stream further ensured the rapid distribution throughout the whole culture of the food added periodically, and evaporated water from the culture, the rate of flow of air being so adjusted that the water removed just balanced that added in the food solution, thus keeping the culture volume constant. The dipstick referred to above enabled the volume to be checked within ± 2%. However, at the lowest temperature investigated (15°) the rate of evaporation was insufficient, with any convenient air flow, to compensate for the food addition; the bacterial counts have therefore been corrected for the small average daily increase in culture volume.

Another essential function of the air stream was to keep the culture adequately oxygenated. The continuous bubbling also prevented the formation of a sediment of cells, the existence of which might have invalidated the cell counts.

Control of pH. Preliminary experiments having shown that in weakly buffered media alkaline metabolic products soon raised the pH to an inhibitory level, the cultures were buffered at pH 7·0 with M/30-phosphate. This sufficed to hold the pH reasonably constant throughout the longest experiments, which exceeded 30 days. The final pH was never above 7·4; other work has shown that a change of this magnitude has little effect on the growth of these cultures.

Temperature control. The culture flask was immersed in a thermostatically controlled water-bath up to the unions of the necks with the flask. The temperature variation did not exceed ± 0·1°.

Procedure at the beginning of an experiment. 1550 ml. of the phosphate buffer were placed in the culture flask and the whole autoclaved, the final volume being 1450 ml. The stock food solution was autoclaved in a 3 l. Pyrex reservoir flask. All connecting parts such as the food jet, level indicator, syringe mechanism, etc., were wrapped separately in paper and sterilized in a 'drying' autoclave. When cool, the culture flask was placed in the water-bath at the desired temperature, the various parts of the apparatus fitted together aseptically, and the air stream started.

After temperature equilibrium had been reached the inoculum was added to the flask and the syringe mechanism controlling the food supply set in operation. Inoculation was at the rate of 300–400 cells/ml. of buffer; this was achieved by adding 5 ml. of a suitable dilution in sterile water of a 24 hr. Difco nutrient broth culture of *Bact. coli* type I grown at 35°. Samples (2·5 ml.) of culture were withdrawn from the culture flask for estimation of the numbers of total and viable cells.

Method of viable counting. The usual plating technique was employed, the culture sample being diluted in sterile M/30-phosphate buffer solution. Eight replicate plates on Difco nutrient agar were made from a suitable dilution of each sample and these were incubated at the temperature of the experiment concerned, except when this was 15°, when an incubation temperature of 20° was used in order to accelerate growth of the colonies.

Estimation of total cells. The turbidity of the sample was measured in a Spekker photoelectric absorptiometer and the results converted to numbers of cells by means of a factor established from special calibration experiments. The latter were conducted at 35° as described above, and the turbidity was determined on samples which were also used for direct microscopic counts. A Thoma counting chamber 0·02 mm. deep was employed, using a $\frac{1}{8}$ in. objective and an 18× compensating ocular. The organisms were killed and stained to render them more easily visible by the addition of 0·5 ml. of 0·1% aqueous acriflavine to 5 ml. of culture. Clumps of cells were very rare and small, and when seen were counted as one unit. Paired cells were infrequent but were counted as one unit unless obviously on the point of separating.

A large number of statistically satisfactory direct counts were obtained, the standard errors of which varied from ±2 to ±6% according to the number of organisms counted (Fisher, 1938). It was then found that the ratios of the numbers of cells/ml. of the culture to the absorptiometer readings (corrected for the gradually deepening colour of the culture caused by the accumulation of the yellow-brown coloured material of the broth) were scattered about a mean. This had a value of 13·62 million cells/ml./absorptiometer unit, with a standard error of ±0·26 million, i.e. approximately 2%. This method is, however, not suitable for estimating extremely low numbers of cells, and total cell counts based on absorptiometer readings of 1 unit or less are therefore only approximate.

EXPERIMENTAL AND RESULTS

Experiments performed. Experiments were carried out at temperatures of 15°, 20°, 25°, 30°, 35° and 40°, daily estimations being made of the numbers of total and viable cells. Only one experiment was performed at each temperature, except at 35°, where the data given are the mean values from several replicate experiments previously reported (Jordan & Jacobs, 1944). Each experiment was continued for at least 22 days, a time amply sufficient to reveal the shape of the growth curve. In two instances, namely, the experiments at 15° and 30°, the food supply was stopped after 17–18 days, but sampling was continued in order that the effects of starvation could be studied. The results obtained are given in Table 1, and the changes in numbers of total and viable cells with time are shown graphically in Figs. 1–3.

General observations

It is evident that the general course of the experiments at all temperatures was similar to that of those previously conducted at 35°, in that an 'initial phase' of varying growth rate was followed by a 'steady phase' of roughly constant rate of increase in total cell count. But whereas in the steady phase of the experiments at 35° the viable count remained approximately constant, in the experiments now reported it increased continually and steadily in all cases except at 40°, in which case there was a tendency for it to decline. It is convenient in describing the influence of temperature on the cultures to consider the initial and steady phases separately; the description of the effects of starvation is given subsequently.

Table 1. *The effect of temperature on the relation between numbers of total cells (T) and viable cells (V) in* Bact. coli *cultures with a constant food supply.*

Cultivation at pH 7·0. Constant rate of food supply: 15·2 mg. dehydrated Difco broth/hr.

Temperature of experiment (°); No. of cells (millions/ml.)

Time (days)	15		20		25		30		35*		40	
	T	*V*	*T*	*V*	*T*	*V*	*T*	*V*	*T*	*V*	*T*	*V*
1	⩽14	<0·0001	—	—	163	17	164	97·6	80	41	204	44
2	⩽14	0·0107	315	171	422	130	344	208	218	119	—	—
3	⩽14	0·638	591	320	629	375	—	—	396	194	409	142
4	268	87·1	825	425	755	506	794	360	514	243	409	174
5	—	—	—	—	—	—	842	415	544	259	422	198
6	1075	484	1222	650	1172	653	903	433	623	281	477	217
7	1286	591	—	—	1385	680	1007	467	658	291	463	209
8	1725	693	1396	872	1450	746	1120	525	665	301	484	220
9	1808	749	1589	910	1580	778	1190	542	649	297	—	—
10	1910	807	1680	987	1730	846	—	—	738	307	477	180
11	2142	968	1880	1046	1785	860	1343	602	800	328	525	186
12	—	—	—	—	—	—	1370	644	804	314	654	178
13	2522	1118	2100	1180	2120	945	1416	600	856	317	687	181
14	2470	1132	2240	1200	2230	993	1504	585	879	314	680	156
15	2660	1250	2360	1254	2480	1088	1540	664	921	301	627	157
16	2782	1290	2610	1320	2390	1026	1566	674	905	284	—	—
17	†2920	†1470	2580	1400	2515	1179	—	—	993	289	695	186
18	2758	1375	2630	1388	2620	1157	†1718	†652	938	298	722	156
19	—	—	—	—	—	—	1650	595	1010	297	—	—
20	2740	1345	2880	1400	2810	1300	1538	627	1057	297	830	186
21	2670	1326	2950	1515	2880	1316	1668	548	1119	283	817	197
22	2530	1382	3100	1600	2940	1340	1554	558	1126	277	912	183
23	2510	1274	—	—	—	—	—	—	1163	282	—	—
24	2550	1352	—	—	—	—	—	—	1186	292	—	—
25	2400	1361	—	—	—	—	1470	540	1222	270	—	—
26	—	—	—	—	—	—	1445	533	1254	250	—	—
27	2420	1320	—	—	—	—	1390	461	1263	246	—	—
28	2440	—	—	—	—	—	1380	378	1255	247	—	—
29	2460	—	—	—	—	—	1342	355	1252	242	—	—
30	2315	—	—	—	—	—	—	—	1348	246	—	—
31	2280	—	—	—	—	—	—	—	1480	258	—	—
32	—	—	—	—	—	—	—	—	1535	260	—	—
33	—	—	—	—	—	—	—	—	1588	255	—	—

* Values given at 35° are the means from several replicate experiments previously reported (see text).

† The food supply was stopped immediately before these counts.

The initial phase

During the initial stages of cultivation the growth rates, measured by the increase in either total or viable populations, varied considerably with time, and the graphs of numbers against time are sigmoid (see Figs. 1–3). The end of the initial phase was determined by inspection as the time (to the nearest day)

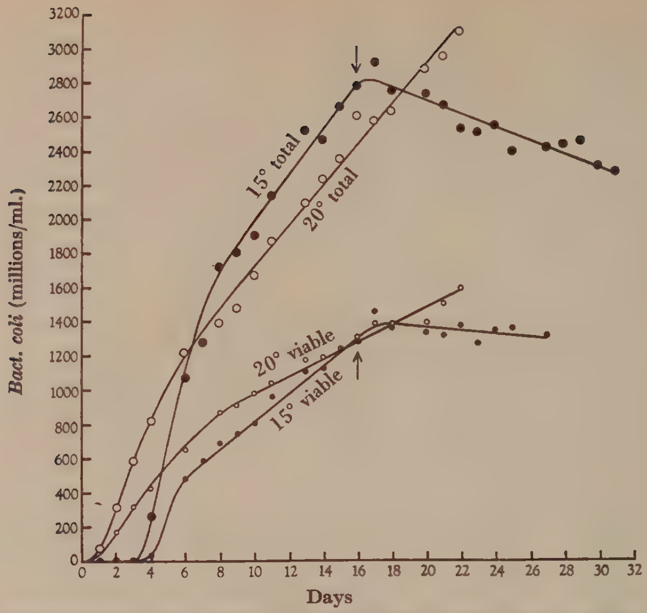

Fig. 1. Growth curves for *Bact. coli* on a constant food supply of 15·2 mg. Difco broth/hr. at 15° (full circles) and 20° (open circles) at pH 7·0. Point at which food supply was stopped is marked by arrow.

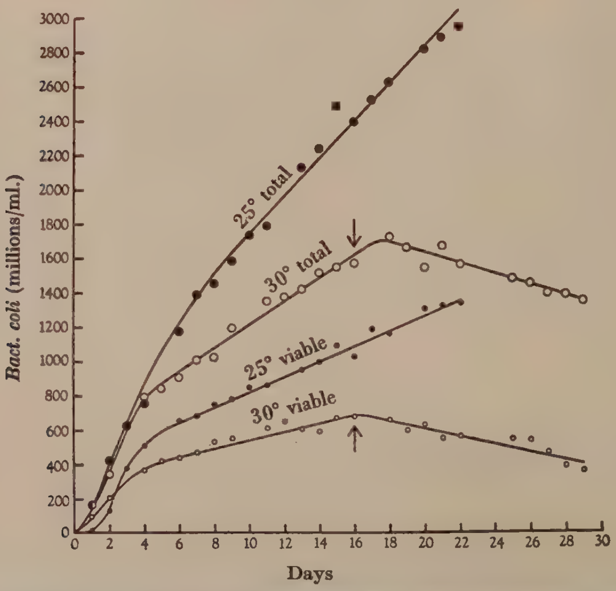

Fig. 2. Growth curves for *Bact. coli* on a constant food supply of 15·2 mg. Difco broth/hr. at 26° (full circles) and 30° (open circles) at pH 7·0. Point at which food supply was stopped is marked by arrow.

at which the sigmoid curve of the initial phase met the linear graph characteristic of the steady phase. It was not always easy to fix the exact time, especially when the growth in the early part of the initial phase had been rapid, because the curves then flattened out slowly and the initial phase merged very gradually into the steady phase. However, by plotting the graphs on a large scale and comparing for each experiment the curves of both total and viable cells, satisfactory times were obtained. The durations of the initial phases are given in Table 2, and it is clear that the length of this phase depended markedly on the temperature, increasing as the latter was lowered.

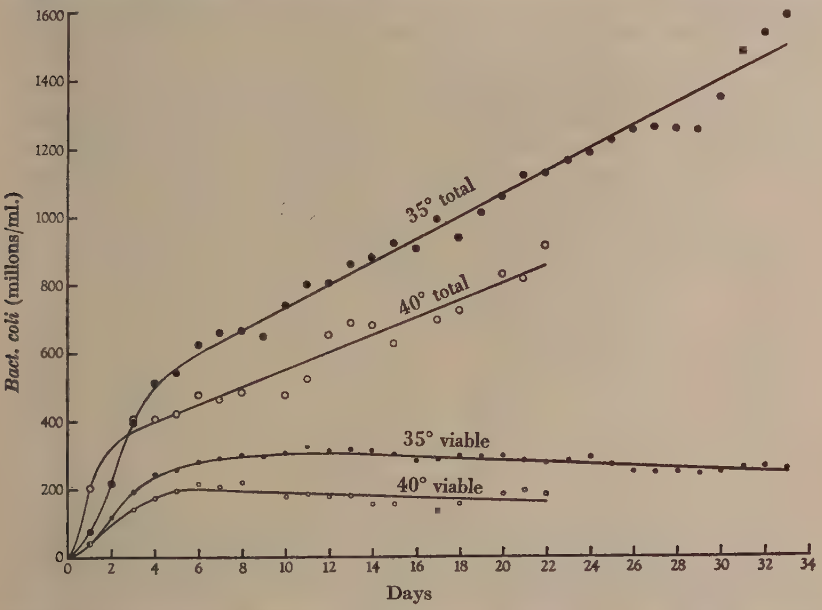

Fig. 3. Growth curves for *Bact. coli* on a constant food supply of 15·2 mg. Difco broth/hr. at 35° (full circles) and 40° (open circles) at pH 7·0.

Table 2. *The effect of temperature on the duration of the initial phase, together with the values of the total and viable populations at its termination, i.e. at start of steady phase*

T_s=total count at start of steady phase (millions/ml.).
V_s=viable count at start of steady phase (millions/ml.).

Temp. (°)	Duration of initial phase (days)	Population at end of initial phase (millions/ml.)		Ratio of total to viable count at end of initial phase
		Total (T_s)	Viable (V_s)	
15	8	1700	670	2·54
20	8	1480	880	1·68
25	7	1410	690	2·04
30	6	945	445	2·12
35	6	595	285	2·09
40	5	425	205	2·07

The effect of temperature on the total cell count at the end of the initial phase (T_s, Table 2) was twofold; there was the anticipated effect of the phase duration, because of the continual addition of food, and also a direct effect of low temperature leading to a further increase in the count. T_s is a measure of cell substance, and clearly more was formed relative to the phase length in a long phase than in a short one. Since the initial phase was longest at the lower temperatures, it follows that the efficiency of conversion of food into cell substance was highest at low temperatures. The fact that in general the viable count at the end of the initial phase (V_s) varied in the same way as T_s is shown by the relative constancy of their ratios to the total counts (Table 2). These ratios are in several cases very close to 2·0; the mean of the six separate values is 2·09, with a standard deviation of ±0·274 and a coefficient of variation of 13·1%, so it is possible that this ratio should have had a value of 2·0 at all the temperatures employed. However, V_s at 15° was lower than at 20°, after initial phases of equal length; this is anomalous in view of the regular increase in V_s with falling temperature between 40° and 20°.

Table 3. *The time required to reach a total population of* 300 *million cells/ml. at various temperatures*

Temperature (°)	Time (days)
15	4·2
20	1·9
25	1·5
30	1·7
35	2·1
40	1·4

The behaviour of the cultures in the initial phase varied considerably according to the conditions. Reliable total cell counts below 200 millions/ml. were secured in only three of the experiments; the shapes of the curves below this point are uncertain. As a basis for comparison of the development of the cultures during the initial phase the time taken to reach a total cell population of 300 millions/ml. has therefore been arbitrarily chosen. This time falls on the well-defined portions of the curves. It has the additional advantage of corresponding roughly to the cell density in a moderately good broth culture grown for about 18 hr. under ordinary conditions, thus affording a means of comparing these cultures with normally grown ones. The times taken to reach 300 million cells/ml. are given in Table 3 and, as would have been expected, development was slow under these conditions of restricted food supply. Clearly, the effect of temperature was relatively slight until the lowest temperature was reached, when there was a marked slowing in the rate of development to this arbitrary population level. As far as can be seen, the same conclusion would have been reached if a much lower level of population had been chosen. This result is in marked contrast with the influence of temperature on the rate of growth of the cultures in the steady phase (see below). When the viable counts are considered similar effects are seen.

The steady phase

As in the experiments previously reported, there was in the subsequent steady phase a tendency for the total counts to increase in a step-wise manner, but at present it is convenient to treat the data as if the rates of cell increase had been constant in each experiment. Also, there was always a marked difference between the total and viable counts, but the major contrast between the present and former (Jordan & Jacobs, 1944) sets of experiments lies in the fact that at all the temperatures employed except one (at 40°) the viable counts instead of remaining approximately constant during the steady phase, showed a marked and continuous increase. At 40° the viable counts in this phase showed a slight tendency to decline. Evidently at pH 7·0 a temperature of about 35° is rather critical for the viability of the cells of these cultures.

Table 4. *The effect of temperature on the relation between numbers of total and viable cells and time in the steady phase*

Temp. (°)	Equation of regression of total count on time $T=T_s+\Delta T\ (t-t_s)$*	Standard error of ΔT	Equation of regression of viable count on time $V=V_s+\Delta V\ (t-t_s)$*	Standard error of ΔV
15	$T=1700+136{\cdot}8\ (t-8{\cdot}0)$	$\pm 7{\cdot}01$	$V=670+83{\cdot}6\ (t-8{\cdot}0)$	$\pm 2{\cdot}95$
20	$1480+118{\cdot}9\ (t-8{\cdot}0)$	$\pm 3{\cdot}86$	$880+50{\cdot}6\ (t-8{\cdot}0)$	$\pm 2{\cdot}23$
25	$1410+108{\cdot}9\ (t-7{\cdot}0)$	$\pm 1{\cdot}17$	$690+44{\cdot}3\ (t-7{\cdot}0)$	$\pm 1{\cdot}48$
30	$945+\ 66{\cdot}8\ (t-6{\cdot}0)$	$\pm 2{\cdot}41$	$445+24{\cdot}4\ (t-6{\cdot}0)$	$\pm 3{\cdot}28$
35	$595+\ 33{\cdot}2\ (t-6{\cdot}0)$	$\pm 0{\cdot}90$	$285+\ 4{\cdot}5\ (t-6{\cdot}0)$	$\pm 1{\cdot}11$
40	$425+\ 25{\cdot}3\ (t-5{\cdot}0)$	$\pm 1{\cdot}82$	$205-\ 2{\cdot}3\ (t-5{\cdot}0)$	$\pm 1{\cdot}17$

* T_s = total count in millions/ml. at start of steady phase,
t = time in days,
t_s = time at which steady phase began,
ΔT = rate of increase in total count in millions/ml./day,
V_s = viable count in millions/ml. at start of steady phase,
ΔV = rate of increase in viable count in millions/ml./day.

If these cultures are treated as if during the steady phase there had been regular daily increments in both total and viable counts, it becomes logical to represent these increments by the slopes of the lines of regression of numbers of cells on time. The equations of these lines have been calculated by the usual method of least squares and are given in Table 4 in a form which makes it plain that the lines originate from the points which define the end of the initial phase or, what is the same thing, the beginning of the steady phase (see footnote to Table 4). The straight lines in Figs. 1–3 correspond to these equations. The standard errors of the slopes of these lines are also given; they are seen to be relatively small, but it must be remembered that if the phenomenon of step-wise increments is real these standard errors have little meaning. It may be mentioned that in the steady phase at 35° the viable counts were only roughly constant (Jordan & Jacobs, 1944), and a closer approximation to the actual condition is given by a bilinear treatment as shown in Fig. 3. There was first a slow increase followed by a slow decline. The regression equation during this decline has not been given in Table 4 since this line does not pass through the point defining the end of the initial phase; the equation therefore cannot be put in the same form as the others in the table. Its equation is $V = 351{\cdot}5 - 3{\cdot}28t$, and the standard error of the regression coefficient is $\pm 0{\cdot}44$.

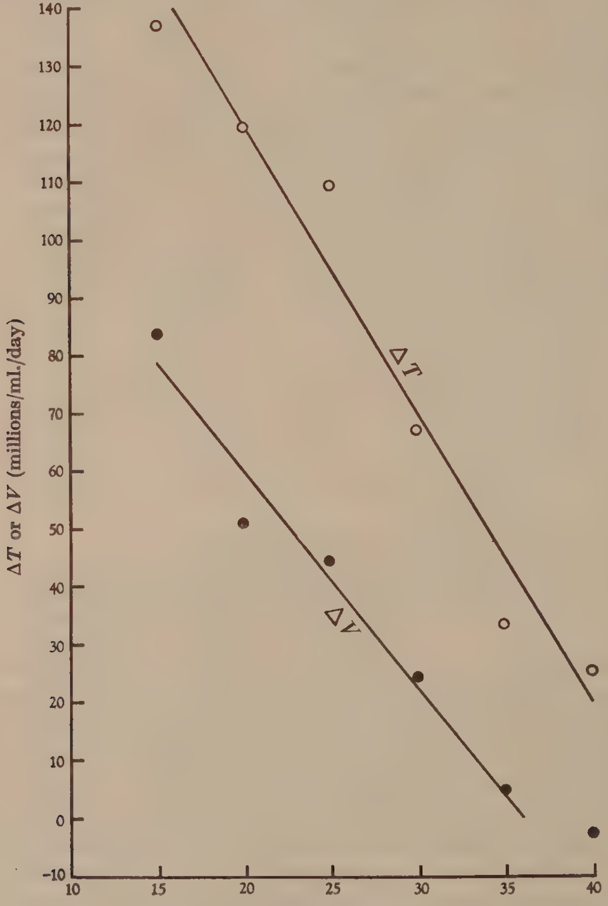

Fig. 4. Relation between daily increment in total cells (ΔT) and viable cells (ΔV) and temperature at pH 7·0.

From Table 4 it is clear that temperature had a marked effect on the rate of formation of viable cells. The striking fact is that the lower the temperature the higher was the rate, and there was an almost exactly linear relationship between temperature and rate of formation between 15° and 35°. This is shown in Fig. 4, in which the line drawn is the calculated best-fitting straight line whose slope is $-3{\cdot}69 \pm 0{\cdot}41$. As shown by this graph the rate of increase in the viable count would have become zero at just over 36° and, if the linear relationship had continued to hold, a further increase in temperature should have led to a declining viable population. This was actually the case at 40° (see Table 4), but the rate of decrease was small and much below that anticipated from the linear relationship below 35°.

In these cultures the total cell counts were always much greater than the viable counts. A high rate of increase in the viable count would naturally lead to a high rate of increase in the total count, and this is in fact observed. Although low temperature favoured a high rate of increase in total cells the rate of increase did not reach zero at 40°, where there was a decrease in viable cells. The relationship between temperature and rate of increase in total cells (ΔT) was linear as shown in Fig. 4, where the line drawn is the calculated best-fitting straight line whose slope is $-4{\cdot}90 \pm 0{\cdot}50$. This line indicates that ΔT should be zero at a temperature of 44·2°, which would thus seem to be a limiting temperature for growth. The best condition for high rate of formation of total cells was, therefore, like that for high rate of formation of viable cells, a low temperature.

The effects of starvation

Under the conditions of starvation which obtained after the food supply had been stopped, the total and viable counts both fluctuated considerably (Figs. 1, 2). The fluctuations sometimes suggested that step-wise decrements had occurred, but for convenience the changes have been assumed to be linear with time and the slopes of the calculated lines of regression are given in Table 5.

Table 5. *The effect of temperature on the daily changes in the total cells* (ΔT) *and viable cells* (ΔV) *in starved cultures*

Temp. (°)	ΔT (millions/ml./day)	ΔV (millions/ml./day)
15	$-38{\cdot}2 \pm 4{\cdot}37$	$-10{\cdot}0 \pm 4{\cdot}96$
30	$-31{\cdot}5 \pm 3{\cdot}56$	$-22{\cdot}8 \pm 3{\cdot}45$

The total counts declined sharply at both temperatures and more rapidly at the lower, but in the case of the viable cells the higher the temperature the faster was the decline. There was no evidence that the viable counts would have reached a constant low level of about 100 million cells/ml. as they did in the experiments at 35° cited above, but the observations were not sufficiently prolonged.

DISCUSSION

In the cultures studied here so few cells were originally present that the rate of food utilization must at first have been less than that of its addition. The critical food level, at which growth as measured by cell division ceases to be dependent on its concentration (Penfold & Norris, 1912), may even have been exceeded. Where food concentration does not limit the growth-rate the points obtained by plotting the logarithms of the numbers of viable cells against time should fall on a straight line. At 15° this was so for a considerable time (Fig. 5), but at all other temperatures food was a limiting factor by the first day after inoculation. Since the cells of the inoculum were added to a fluid virtually devoid of nutrients there may have been an inactive period in addition to the usual lag phase in which increase of cell size occurs without cell division. From Fig. 5, by extrapolation backwards according to the method of Lodge & Hinshelwood (1943), the total lag phase at 15° is found to have been slightly over 1 day. At all other temperatures it must have been much less. The total lag having been so short, an inactive period could not have constituted an important phase in these cultures. A period of increase in cell size probably occurred in the early part of the initial phases of the present experiments, since there was an abnormally high ratio of total to viable count at this stage. The ratios calculated for the earliest time in each experiment at which simultaneous total and viable counts were secured are given in the upper part of Table 6 in order of decreasing magnitude. The ratio yielded by the composite data for 35° has not been included because, as explained previously (Jordan & Jacobs, 1944), the counts given at one day (see Table 1) do not reflect the true condition of the individual experiments at that time. In general the lower the viable count the higher is the ratio; not until the former reached 50–100 million cells/ml. did the latter approach the range of 1·68–2·54 characteristic of the end of the initial phase (see Table 2). The individual experiments at 35° also support this finding. Therefore, unless many cells were non-viable, the individuals must have been abnormally large. A high ratio of total to viable cells persisted longer at 15° than at any other temperature (see the lower half of Table 6). Further, the viable count at the end of the initial phase at 15° was unexpectedly low, and these two observations both indicate that at this temperature the so-called normal proportionality between total cells and turbidity may never have been attained. If this were so, the recorded number

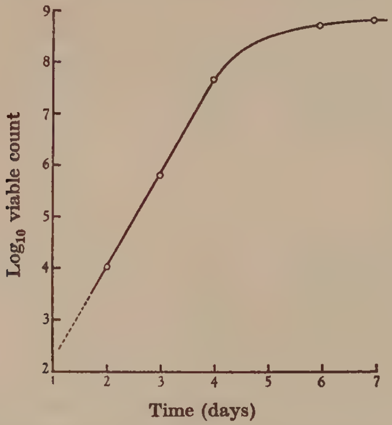

Fig. 5. Relation between log viable count and time in the initial phase at 15° and pH 7·0.

of total cells at the end of the initial phase at 15° must be larger than the actual number and the ratio T_s/V_s too high. If the value for 15° be excluded, the five remaining values of T_s/V_s in Table 2 have a mean of $2{\cdot}00 \pm 0{\cdot}08$. Apparently, over a wide range of temperature, about half the cells present at the end of the initial phase were incapable of further division. The graphs in Figs. 1–3, purporting to represent changes in total cell numbers with time, ought strictly to be regarded as curves of changes in total cell substance, but it has been found convenient to retain the expression 'numbers of total cells' although it is realized that at 15° the numbers referred to may be too high.

Table 6. *The earliest simultaneous values of the total and viable counts in the initial phase of each experiment, and the total and viable counts at the earliest comparable time in all experiments, with their ratios*

Temp. (°)	Time (days)	Total count (millions/ml.)	Viable count (millions/ml.)	Ratio of total to viable count
25	1	163	17	9·6
15	4	268	37	7·2
40	1	204	44	3·9
20	2	315	171	1·8
30	1	164	98	1·7
15	4	268	37	7·2
20	4	825	425	1·9
25	4	755	506	1·5
30	4	794	360	2·2
35	4	514	243	2·1
40	4	409	174	2·4

The view has already been put forward (Jordan & Jacobs, 1944) that at the end of the initial phase the cultures became stabilized in the sense that any food which may have accumulated in the early stages had been used up, so that further additions were consumed as fast as they were supplied, a condition which resulted in the so-called steady phase. Under these conditions the numbers of both total and viable cells appeared to show a distinct tendency to increase in a step-wise manner (see Figs. 1–3). Other workers have recorded that in the logarithmic phase of ordinary cultures growth may proceed in waves (Wilson & Miles, 1946). At present a convenient and satisfactory approximation is to treat these cultures as if there had been regular daily increments in numbers during this phase. The increments (ΔT and ΔV for the total and viable counts respectively) are measures of the rates of growth of the cultures as a whole and do not reflect the division rates of the individual cells. Evidently, the numbers of total and viable cells formed from a fixed amount of food remained approximately constant irrespective of the age of the culture, i.e. of the number of cells composing it. Bail (1929) suggested that for any given culture and conditions of growth there is a maximum population which can be supported. The experiments reported here are in agreement with this view in so far as the numbers of viable cells are concerned, but only provided that the temperature of incubation is not too far removed from that ordinarily considered to be the optimum for the organism employed (in this case 37°). If the total amount of cell substance is regarded as the 'population' in these cultures, then there appears to be no indication, at any of the temperatures used, of the existence of Bail's maximum population.

Changes in temperature markedly affected ΔT, which was highest when the temperature was lowest and, as there was a constant food supply, it is evident that the efficiency of conversion of the food into cell substance increased with decreasing temperature. This appears at first sight to be irreconcilable with the usual conception of the optimum temperature for growth. But the latter is normally obtained from cultures growing in the presence of a large excess of food and really represents the temperature at which the mean generation time of the cells is minimal, no account being taken of the efficiency of the process. It is indicated below that, with decreasing temperature, the amount of food wasted by the cells in reactions not leading to synthesis of cell substance declined. With a limited food supply this must have led to an increase in the overall efficiency. The relative amounts of different enzymes in the cells may have varied with the growth temperature (Gale, 1940), and this would have far-reaching effects. In the initial phase, however, variation of temperature within wide limits did not affect the rate of development of the cultures to an arbitrary population level, and the two measures of the growth of the cultures are therefore not comparable. As shown above, it can be deduced that 44·2° is a limiting value above which, in these particular conditions, no growth would have occurred. It is of course true that the type I *Bact. coli* used in this work will grow rapidly at 44° in other nutritional conditions; it is Eijkman-positive and grows and forms gas from lactose at that temperature in MacConkey's broth.

The magnitude of ΔV was also very dependent on the temperature, being greatest when the latter was low. High levels of ΔV would naturally tend towards large values of ΔT, and, in fact, there was a strong tendency in that direction as can be seen from Table 4. Table 7, however, shows that ΔV became larger relative to ΔT as the temperature decreased. The ratio of ΔV to ΔT is a measure of the extent to which the cells formed retained their viability, but it is not the best measure of this property, since it was not true that no growth occurred in the cultures in which ΔV was zero. A better measure is the non-viability index (*n.v.i.*) which has been defined (Jordan & Jacobs, 1944) as the proportion of the cells formed in a generation which is non-viable; this can be calculated in the following manner. The true generation time at any moment in this phase is the time required for a number of viable cells (V) to form V additional cells. Some of these were viable and others not, but they all contributed to the increase in total cells and required for their formation $V/\Delta T$ days. The increase in viable cells was therefore $V\Delta V/\Delta T$, and the actual number of viable cells at the end of this time was thus $V(1+\Delta V/\Delta T)$. If all the cells formed had been viable, the expected number of viable cells should have been $2V$, and the value of the *n.v.i.* is therefore $\frac{1}{2}(1-\Delta V/\Delta T)$. These values are given in Table 7 and show that retention of viability was greatest at the lower temperatures, the index increasing sharply above 30°. The value at 15° may in reality have been even lower than appears from Table 7. Since at this temperature the individual cells were apparently larger than normal, the recorded value of ΔT must be greater than it actually was. Clearly, in this steady phase the food available per cell did not affect the *n.v.i.*, since the latter was constant while the former diminished with time, and the cultures with the densest populations, and therefore the lower food supply per cell, had the lowest indices. Temperature was thus the important factor controlling the *n.v.i.* In connexion with the favourable effect of the lower temperatures on viability as well as on the rate of formation of cell substance, it may be recalled that Thompson (1942) pointed out that there is a wealth of microbial life in cold seas compared with that found in tropic waters.

Table 7. *The effect of temperature on the ratio between the daily increments in viable and total cells* ($\Delta V/\Delta T$), *and on the non-viability index* (*n.v.i.*) *during the steady phase*

Temp. (°)	$\Delta V/\Delta T$	*n.v.i*
15	0·61	0·194
20	0·43	0·287
25	0·41	0·297
30	0·36	0·318
35	0·14	0·434
40	−0·09	0·545

Further experimentation would seem to be desirable to establish firmly whether or no there is, as the data suggest, a temperature zone above 35° within which ΔV remains approximately constant at about zero. If it exists, its range must be limited, as above a certain point thermal death would occur; indeed, non-viability may perhaps be regarded as thermal death occurring at a temperature at which growth of cell substance is also possible. The upper limit of the zone might be 44°, the temperature indicated above as the maximum for growth under these experimental conditions. It is noteworthy that the results of experiments on the thermal disinfection of similar cultures of *Bact. coli* are in agreement with 44° as the minimum temperature at which heat disinfection could occur (Jordan, Jacobs & Davies, 1947).

The method of calculating the amounts of food used at 35° in the construction of a new bacterial cell and in its maintenance, employed previously (Jordan & Jacobs, 1944), cannot be applied at other temperatures as it depended on the constancy of the viable count in the steady phase, and this condition was not fulfilled. There appears to be no ready method of assessing the food used for these purposes. It may be that the amount of food used to form a new cell is substantially the same at all temperatures. If, however, in any conditions the use of the figure of $1{\cdot}083 \times 10^{-9}$ mg. dehydrated Difco broth per cell as calculated for 35° (Jordan & Jacobs, 1944) should lead to an estimate of the food used for new cell formation in excess of the amount actually supplied in any interval, this would indicate that a change in cellular composition or metabolic reactions had occurred. Using this figure, at the largest observed value of ΔT, only 214·8 mg. of food for cell production was required daily, whereas the amount added was 364·8 mg. But the amount of 150·0 mg. apparently available on this basis for maintenance and wastage would have been quite inadequate if the viable cells present were all using food for the latter purposes at the rate of $0{\cdot}456 \times 10^{-9}$ mg./cell/day calculated for 35°, and the requirement at 15° must have been considerably smaller than this.

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A Preliminary Study of the Formation, Assay and Stability of Tyrothricin

By J. C. APPLEBY, E. KNOWLES, J. PEARSON and T. WHITE
The Research Laboratories, John Wyeth and Bro. Ltd., London

SUMMARY: Strain selection and the addition of yeast extract to the medium have enabled high yields of tyrothricin to be produced from the culture fluids of *Bacillus brevis*. Contrary to the accepted view, this organism does ferment carbohydrates with the production of acid, and a description of the characters of the organism is given. A method of assaying tyrothricin has been developed and used to investigate the stability of aqueous solutions of the material. Tyrothricin produced in aerated submerged culture appears to be more stable in solution than that produced by surface culture.

The occurrence of the antibacterial agent tyrothricin in the culture fluids of an aerobic spore-forming bacillus was first recorded by Dubos (1939 *a*, *b*, *c*). A series of further publications (Dubos, 1940, 1941; Dubos & Cattaneo, 1939; Dubos & Hotchkiss, 1941, 1942; Dubos, Hotchkiss & Coburn, 1942; Hotchkiss, 1941; Hotchkiss & Dubos, 1940 *a*, *b*, *c*, 1941) showed that the material was of value as a chemotherapeutic agent and consisted of two clearly defined components—gramicidin and tyrocidine—both polypeptide in nature. These authors discussed the production of the antibiotic in surface culture, described its isolation and purification, and identified as *Bacillus brevis* the organism responsible for tyrothricin formation.

Generally, the bacillus was grown on shallow layers of culture media containing casein hydrolysate, peptone or Tryptone; yields of tyrothricin up to 0·5 g./l. culture fluid were obtained. Optimum yields were given by a strain (B.G.) grown on Tryptone media. A related product, gramicidin S, was reported by Gause & Brahznikova (1944) as being produced in surface culture by another organism of the *B. brevis* type. Numerous other investigations dealing with the chemistry and clinical applications of these materials were reviewed by Hotchkiss (1944). Of these later publications two only were concerned with the conditions of formation of tyrothricin by *B. brevis*. Lewis, Dimick & Feustel (1945) studied the formation of tyrothricin in surface culture, particularly in media based on vegetable waste, while Stokes & Woodward (1943) reported on the production of tyrothricin under conditions of aerated submerged culture. From these and the preceding papers it is clear that under conditions of surface culture *B. brevis* grows well and produces tyrothricin readily in media containing complex nitrogenous nutrients of the peptone, Tryptone and vegetable waste type. The substitution of simple nitrogen sources such as inorganic ammonium salts resulted in sparse growth and poor yields of tyrothricin.

In aerated submerged culture the organism grew well in the presence of complex nitrogen sources but normally failed to produce tyrothricin. Stokes

& Woodward (1943) succeeded, however, in developing a synthetic medium containing glucose, inorganic salts, and an amino-acid as a nitrogen source, which not only supported growth of *B. brevis* but permitted production of tyrothricin in aerated submerged culture. Certain amino-acids were unsuitable for this purpose, and mixtures of amino-acids inhibited tyrothricin formation although they permitted it in surface culture. In general, with suitable single amino-acids, yields of tyrothricin of the order of 0·1–0·3 g./l. culture medium were obtained at an aeration rate of 1·5 l. air/l. medium/min. It was clear from these results that the metabolism of the organism could follow different paths in surface and in aerated submerged culture.

In view of the clinical importance of tyrothricin and the availability of appropriate equipment it was decided to investigate further the possibility of producing the antibiotic by the submerged culture technique. The results obtained are dealt with in the succeeding communication (Appleby, Knowles, McAllister, Pearson & White, 1947). Initially, using the Dubos B.G. strain, a brief survey was made of the production of tyrothricin in surface culture and, as an aid to further work, a rapid method of assay was developed. This method was used to investigate the stability of tyrothricin solutions as prepared for clinical use. The present communication describes the results of this work.

EXPERIMENTAL

Characters of the organism

The organism used in this work, the Dubos B.G. strain of *B. brevis* brought back from the U.S.A. by one of us (T. W.), does not agree in character with the incomplete description of *B. brevis* recorded by Bergey *et al.* (1939), nor with the accounts of this species recorded in previous literature. The most notable discrepancy is in carbohydrate fermentation, as, although *B. brevis* is generally considered to be inert in this respect, we have found that our strain, and strains from the National Collection of Type Cultures, are capable of fermenting a wide range of carbohydrates. We accordingly include a description of the characters of this species as we have found them. Unless otherwise stated, cultures were grown on Difco nutrient agar.

Morphology. Gram-negative motile rod, 0·5 × 2·5–5 μ., sporulating readily. Spores oval, subterminal, and considerably wider than the vegetative cell. A notable feature of cultures is the large number of free spores which stain Gram-positive and present a swollen and granular appearance. These are evidently not resting forms and may give a misleading impression of a mixed culture. They have not been seen in cultures of other strains of *B. brevis*. The organism is capable of extreme pleomorphism in aerated synthetic media.

Plate colonies. At 24 hr. these are about 1 mm. diameter, round, convex, smooth and shining. After 48 hr. the surface becomes drier and finely granular, and the margin irregular. From 3 days onwards there are two distinct zones: (*a*) an internal zone of 1–2 mm. diameter which is raised, dry, finely granular and surrounded by (*b*) a flatter, smooth, moist area of growth, sometimes concentrically ringed, the margin deeply lobed or crenated. No stable colonies with a smooth surface have been obtained and colonial types have remained very constant during 18 months. Four strains from the National Collection of Type Cultures showed a series of gradations between colony forms identical with those of our strains, and a completely smooth colony.

Growth requirements. All strains tested are obligate aerobes with a temperature optimum of 37° and a wide range for growth.

Pigmentation. Cultures are not normally pigmented but may develop a pink pigment on some media containing vitamins of the B group.

Biochemical characters. The organisms produce acid without gas from: glucose, fructose, galactose, sucrose, lactose, maltose, xylose, mannitol, arabinose, sorbitol, salicin and glycogen. Raffinose, inositol, adonitol, aesculin and starch are not fermented. One strain (N.C.T.C. no. 2611), in an unstable smooth colony phase, failed to ferment xylose, mannitol, sorbitol or glycogen. Catalase +; indole −; H_2S +; nitrate reduction +; gelatin liquefaction, saccate becoming stratiform. Litmus milk: sweet curdling and peptonization, the latter complete after about 7 days at 37°.

The basal medium for determination of the sugar reactions consisted of 0·5 % (w/v) proteose-peptone with 0·5 % (w/v) NaCl in tap water at pH 7·8. It was found that, from several peptones investigated, including Difco nutrient broth, the organism was able to produce small amounts of acid without the addition of any carbohydrate. In a 0·5 % solution of the proteose-peptone finally used the pH decreased by not more than 0·8 unit after 3 days' incubation when inoculated from a carbohydrate-free medium. The sugar broths were accordingly made sufficiently alkaline to compensate for this. In some cases the pH was determined electrometrically after inoculation and incubation to confirm acid production. Carbohydrate solutions were sterilized by Seitz filtration (except aesculin, sterilized at 100° for 25 min.; starch and glycogen, sterilized at 110° for 10 min.) and added aseptically to the basal broth to give a final concentration of about 1 % (w/v). Inoculations were made from 24 hr. peptone-water cultures.

It is known that *B. brevis* is actively proteolytic. It is conceivable therefore that previous failure to demonstrate saccharolysis may have been due to the simultaneous production of alkaline substances, and it would appear that the nitrogen source, initial pH, and sensitivity of the indicator may all be factors of importance in this respect. That glucose is in fact utilized has been shown by chemical analysis of cultures (Appleby *et al.* 1947).

Maintenance of cultures

It was found possible, by colony selection, to obtain strains varying markedly in the amount of tyrothricin they produced, either in surface or submerged culture, but with no other distinguishing feature. Thus, of six strains A–F, strain C when first isolated produced twice the amount produced by strain A; strain F was still more efficient and has remained the most satisfactory; while strain D at first produced comparatively little tyrothricin and none at all after 18 months. In order to maintain the activity of strains, bimonthly transfers were made on nutrient agar (1% (w/v) peptone, 0·5% (w/v) NaCl); after incubation for 3 days the cultures were kept at 4°.

Tyrothricin from surface cultures

Surface cultures were grown for 3 days in flat bottles each containing 100 ml. broth (1% (w/v) peptone, 0·5% (w/v) NaCl with or without the addition of 0·3% (w/v) yeast extract (Difco) at pH 6·5). For the inoculum the whole of the growth of a 3-day slope culture, suspended in saline, was subcultured in 100 ml. broth contained in a 250 ml. Erlenmeyer flask. After 2 days' incubation 1 ml. was used as the inoculum for each bottle. These were incubated horizontally to give the maximum surface area with a depth of about 1 cm.

Tyrothricin was isolated in each experiment by pooling the contents of a batch of bottles, acidifying to pH 4·1, and centrifuging off the bacterial cells after standing at room temperature for 24 hr. The separated cells were then extracted for 24 hr. at room temperature with 95% ethanol (50 ml./l. original culture fluid), the ethanolic extract clarified by centrifuging and then poured into 10 vol. 1% (w/v) NaCl solution. The precipitated tyrothricin was centrifuged off after standing for 24 hr., dried *in vacuo* over P_2O_5, and the dried powder defatted with dry ether (8 ml./g. crude product) at 0°. This normally removed some 15% of the weight of the crude product as fatty material. The ether was filtered off and the purified tyrothricin washed with the minimum of dry ether at 0° and again dried out *in vacuo*. The product had a light colour and gave brown solutions in ethanol. The addition of traces of glycerol to the ethanol markedly aided solution of the material.

In general, the results obtained conformed with those reported by earlier workers. In media containing only peptone and NaCl, average strains of *B. brevis* produced *c.* 0·25 g. tyrothricin/l. culture fluid when grown for 72 hr. at 37°. We were able, however, to increase this yield appreciably by isolating strains from the parent B.G. culture as mentioned above, and by using media containing 0·3% Difco yeast extract in addition to the peptone; under these conditions one of the strains (F) gave 0·8 g./l. in 72 hr. Generally the isolated strains appeared each to maintain a characteristic level of tyrothricin yield in a given medium, and in later experiments strain F was found to be particularly good in submerged culture also. The stimulating influence of yeast extract on tyrothricin was, of course, not unexpected, but it does not appear to have been recorded previously, and from the work reported later it appears to be due mainly to the presence of biotin in the extract. This is in agreement with Landy, Dicken, Bicking & Mitchell (1942), who observed that biotin stimulated the growth of *B. brevis*.

Method of assay

The above experiments provided sufficient material to permit the development of a method of assaying tyrothricin. Tishler, Stokes, Trenner & Conn (1941) estimated the separated gramicidin and tyrocidine components by determining the quantities of each required to destroy 50% of the cells of a *Micrococcus* in 2 hr. under standard conditions, the number of surviving cells being determined by plating. Stokes & Woodward (1943) used a modified form of this method. Dimick (1943) assayed tyrothricin solutions by estimating photoelectrically the degree of haemolysis of rat erythrocyte suspensions under controlled conditions. It was felt desirable, however, to develop a method which was rapid and based on the antibacterial activity of tyrothricin rather than on some other property, which could be used without adopting a rigid aseptic technique, and which could be applied for following the course of submerged culture fermentations. These criteria were ultimately realized.

In general, solutions for assay were made up by dissolving 1 g. tyrothricin in 40 ml. 95 % ethanol and diluting with sterile distilled water to a concentration of 25 μg./ml. In the case of culture fluids, 50 ml. samples were acidified to pH 4·1 in centrifuge tubes, the cells centrifuged down after 2 hr. and, after removal of the supernatant, triturated with 5 ml. 95 % ethanol. The suspensions were kept at room temperature for 30 min., recentrifuged, and 1 ml. aliquots of the clear ethanolic supernatants added to 50 ml. sterile distilled water to give the preliminary dilutions for assay.

The test organism was a member of the *Streptococcus lactis* group, supplied by Prof. Raistrick for diplococcin production. After a few weeks in the laboratory, this strain grew well at 37°. It was markedly inhibited by tyrothricin, and the latter was conveniently estimated by its suppression of acid production from glucose by the above organism.

The medium used for assay purposes was a papain digest of ox heart, diluted to contain 0·5 % oxidizable matter, with 1 % (w/v) glucose and sufficient bromcresol-purple to give a distinct colour, at pH 7·0. Stock cultures of the test organism in chalk-litmus milk were kept at 4° and subcultured at fortnightly intervals. From these, further cultures were prepared in litmus milk, incubated at 28° until growth was shown by reduction of litmus with little or no acid production (approx. 16 hr.), and used immediately. With the culture thus in the early logarithmic growth phase, 1 ml. added to 40 ml. heart broth served as the inoculum for assays.

Into each of a series of test-tubes ($3 \times \frac{3}{8}$ in.) were delivered in this order: (*a*) a known amount of tyrothricin solution, (*b*) sufficient sterile distilled water to bring the volume to 1·0 ml., (*c*) 1·0 ml. inoculated broth. This was easily accomplished by using specially designed dropping burettes, each calibrated to deliver 25 drops/ml. of a particular fluid. After mixing, the tubes were immersed in racks in a thermostatically controlled water-bath at 37°. If, as was generally arranged in practice, the difference between the tyrothricin content of two successive tubes of a series was one drop of a solution containing *c.* 25 μg./ml. tyrothricin, the strength of an unknown solution (by comparison with a standard solution) could be estimated to within 0·5 μg./ml. Two control tubes containing (*a*) no tyrothricin, (*b*) 25 μg. tyrothricin were included in each set.

The end-point was taken as the first tube in which the purple colour remained after incubation at 37° for 4 hr., the readings being made at the time when the control series first gave clear-cut results. This did not indicate complete bacteriostasis but provided a means of assessing the bacteriostatic value of an unknown solution by comparison with one of known strength. Incubation below 37° or at a temperature not sufficiently uniform, or failure to incubate the assay tubes immediately after mixing the inoculum with the tyrothricin, tended to result in intermediate colour changes; the end-point under these conditions was less clear-cut. By adhering rigidly to a standardized technique it was found that assay results by this method were surprisingly constant for a solution of known strength, not only over a long period, but also with different workers. Differences in the treatment of the test culture did cause some variation in assay values, so a control of standard strength was always included. Using the above technique, inhibition of acid production was usually caused by 7·0 μg. tyrothricin per tube.

In some cases it was not possible to prepare an extract of tyrothricin from a *B. brevis* culture, but was necessary to estimate the approximate amount of antibiotic in the culture fluid itself. The sample of culture was then centrifuged to remove the majority of the cells, and the supernatant itself assayed as above but using a medium containing cysteine hydrochloride. The presence of 0·1 % (w/v) of this substance usually prevented growth of *B. brevis* under the test conditions (especially if the surface of the culture was sealed with sterile liquid paraffin), and 0·5 % inhibited *B. brevis* completely but had no adverse effect on the test organism

Stability of tyrothricin solutions

Certain early publications give the impression that tyrothricin solutions gradually lose antibacterial activity through temperature inactivation and, in consequence, it is generally advised that for clinical application freshly prepared solutions should be used. Dubos & Hotchkiss (1942) showed, however, that although aqueous tyrothricin solutions lost activity on heating or long standing, this was not a true inactivation but was due to colloidal aggregation followed by precipitation of the antibiotic. The full activity could be restored by redissolving the precipitated material in ethanol and resuspending in colloidal form by adding this extract to appropriate quantities of distilled water.

Table 1. *Percentage loss of antibacterial activity of surface-culture tyrothricin solutions upon heating*

Concentration of solution (µg./ml.)	Temperature of heating (°)	% loss of activity after heating for		
		10 min.	60 min.	24 hr.
25	37	12·5	22·3	36·3
	50	12·5	30·0	68·2
500	37	12·5	22·3	41·7
	50	12·5	30·0	70·7
5000	37	22·3	30·0	63·2
	50	41·7	75·0	82·5

Since a tendency to loss of activity is clearly of importance in clinical treatment and the way in which the material is used, some investigation of this matter was carried out by comparing the surface-culture material already discussed with material produced by submerged culture (Appleby *et al.* 1947). Aqueous solutions of both types of products from surface and submerged cultures at 25 µg./ml. were prepared by appropriate dilution of solutions (1·25 g. tyrothricin/100 ml. ethanol), and maintained in sealed Pyrex tubes at room temperature for several months with periodic readings of turbidity. A clear distinction emerged in that the initial turbidities (Spekker, 1 cm. cell) were: 'surface' material, 0·136; 'submerged' material, 0·054; indicating presumably a smaller particle size or greater water solubility of the 'submerged' product, since no difference was found in the gramicidin/tyrocidine ratio for the two products. It was also found that the initial assay figure for the 'submerged' product was some 10% higher than that of the 'surface' product. On storage, the surface-produced material showed a gradual colloidal aggregation and increased turbidity leading to complete precipitation in less than 3 months. The submerged-culture material, however, showed no sign of precipitation over a period of 6 months, the turbidity during this period increasing to 0·091, which was still well below the initial value for the 'surface' material. In accord with this it was found, by assaying an aqueous solution (25 µg./ml.) of submerged-culture tyrothricin at intervals for 6 months, that solutions prepared as for clinical use showed no detectable loss of antibacterial activity if maintained at 4°. This is clearly a fact of practical value and indicates some difference between the 'surface' and the 'submerged' products.

Some investigation was also carried out of the effect of heat on the inactivation of aqueous tyrothricin solutions of concentration 25, 500 and 5000 μg./ml. Such solutions were maintained at 37° and 50° up to 24 hr. and assayed at intervals for bacteriostatic activity; unheated controls were included in each case. The latter showed no detectable inactivation at room temperature during the period of test. The results of the higher temperature experiments, presented in Table 1 for the 'surface' material, show that the extent of inactivation by heat increases both with the time of heating and with increasing concentration of tyrothricin. Similar results were obtained with submerged-culture tyrothricin, such differences as existed suggesting, if anything, that the latter material was the more stable. The distinction, however, was not pronounced and was certainly not as clear as the findings in the other stability tests.

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The Production of Tyrothricin by Submerged Culture of *Bacillus brevis* in Synthetic Media

By J. C. APPLEBY, E. KNOWLES, R. C. A. McALLISTER,
J. PEARSON and T. WHITE

The Research Laboratories, John Wyeth and Bro. Ltd., London

SUMMARY: Synthetic media containing ammonium succinate as the sole source of nitrogen permit *Bacillus brevis* to grow and form tyrothricin in aerated submerged culture. The yield of the antibiotic is almost doubled by adding a complex of B-vitamins to the medium, the effect being due specifically to the presence of biotin.

Urea can be used as an alternative but less satisfactory source of nitrogen, but ammonium succinate cannot be replaced by ammonium salts of a number of other organic acids. The process can be operated successfully on a large scale.

Normally, *B. brevis* will grow, but will not produce tyrothricin, in submerged culture in the presence of complex nitrogen sources. An exception to this has been observed, but metabolic investigations show distinct differences in the behaviour of the organism (in submerged culture) in synthetic and in peptone media.

An outline of previous work on the submerged-culture formation of tyrothricin has been given in the preceding paper (Appleby, Knowles, Pearson & White, 1947). It was then decided to aim at developing a synthetic medium containing ingredients which would provide *Bacillus brevis* with nitrogen sources capable of supporting the production of tyrothricin in submerged culture, and which would be available in such quantity and at such cost as would permit of the use of the method on a large scale. This aim was realized.

EXPERIMENTAL

Methods

In all experiments 6 l. of medium was made up in a 10 l. aspirator, the lower outlet of which was fitted with a rubber stopper carrying a thermometer, a sampling tube with external cotton-wool cover, and a glass aeration tube extending horizontally into the aspirator about 1 in. above the bottom and terminating in a number of fine jets. This tube was connected to a compressed air line *via* a cotton-wool packed section for air filtration, and a 'Rotameter' for measuring the air flow. The aspirator outlet was closed by a rubber stopper carrying a short vertical $\frac{1}{2}$ in. bore glass tube covered with a wired-on external cotton-wool plug. This tube served for adding the inoculum, for periodic addition of anti-foam (sterilized 2 % (w/v) stearyl alcohol in lard oil), and as an air outlet. The medium was adjusted to the required pH (usually 6·5) before sterilizing, and the whole assembly steam-sterilized at 12 lb./sq.in. pressure for 30 min. After cooling, the pH and the medium constituents were checked and the aspirator inoculated in a 'sterile area' (Grenfell, Legge & White, 1947). Each aspirator was inoculated with 10 ml. from a 3-day peptone-yeast extract broth culture of *B. brevis*, and was then placed in an incubation room at 37° and connected to the compressed air line. Air was supplied at 20 lb. pressure and controlled at a rate of 1 l. free air/l. medium/min., the resultant agitation making stirring unnecessary. Samples were taken at intervals by removing the external cotton-wool plug of the sampling tube, flaming the end of the latter, and then allowing the requisite volume of culture fluid to run out into a sterile sampling flask, the first 20 ml. sample being discarded. Analyses were carried out for pH, glucose, ammonia, turbidity and tyrothricin content. The last named was determined by the assay method of Appleby *et al.* (1947), by direct isolation of tyrothricin, or by both methods. The pH was determined electrometrically, turbidity with a 'Spekker' photoelectric absorptiometer (1 cm. cell), ammonia by micro-Kjeldahl, and glucose by the method of Schaffer & Hartman (1920). Turbidity, glucose and pH determinations were carried out directly on portions of the sample; ammonia determinations were made with samples of clear supernatant after centrifuging off the bacterial cells. Tyrothricin isolations were made by the method described by Appleby *et al.* (1947).

Preliminary experiments

Submerged-culture experiments were carried out with our strain F of the Dubos B.G. strain of *B. brevis* using initially the following media: (*a*) that used previously for surface culture—i.e. peptone 10 g., yeast extract 3 g., NaCl 5 g., water to 1 l., pH 7·0; (*b*) that of Stokes & Woodward (1943) with glutamic acid as the nitrogen source—i.e. glutamic acid 5 g., glucose 10 g., inorganic salts (see below), water to 1 l., pH 6·4; (*c*) as (*b*) with glutamic acid substituted by asparagine (2·5 g./l.); (*d*) as (*b*) with the addition of Difco yeast extract (3 g./l.). In conformity with the results of Stokes & Woodward (1943) good growth occurred, but no tyrothricin was formed in medium (*a*). At a later date, however, our strain F did develop the capacity of producing tyrothricin in peptone media in submerged culture, and this point will be discussed later. The glutamic acid medium (*b*) gave a tyrothricin yield of 0·30 g./l. culture, while the asparagine medium (*c*) gave 0·21 g./l. In medium (*d*) growth of the organism was obviously much greater than in medium (*b*), but the presence of the yeast extract completely inhibited the formation of tyrothricin.

Ammonium salts of organic acids as sources of nitrogen

Attempts were next made to produce tyrothricin by aerated submerged growth of *B. brevis* in media containing ammonium salts of organic acids as the only sources of nitrogen, glucose being used as the primary carbon source with the usual inorganic salts also present. Attention was focused on ammonium succinate, citrate, tartrate, lactate and carbamate, each being used separately at a level of 5 g./l. in a medium containing also (g./l.): glucose 10; K_2HPO_4 0·5; KH_2PO_4 0·5; $MgSO_4,7H_2O$ 0·2; NaCl 0·01; $FeSO_4,7H_2O$ 0·01; $CaCl_2$ 0·25.

Each aspirator was run at 37° with equal volume aeration for at least 150 hr. The ammonium lactate and carbamate media failed to support growth of the organism. Sparse growth occurred in the ammonium tartrate medium, but no tyrothricin could be isolated nor could any antibacterial activity be demonstrated in the culture fluid. Both the ammonium citrate and succinate media permitted good growth, and the analytical data showed appropriate utilization of nutrients. With the former salt no antibacterial activity developed nor could any tyrothricin be isolated. The ammonium succinate medium, on the other hand, showed a progressively increasing antibacterial effect and gave a final yield of tyrothricin of 0·5 g./l. culture. Repetitions of these experiments have consistently given identical results except that occasionally the tyrothricin yield from the ammonium succinate medium fell to 0·3 g./l.

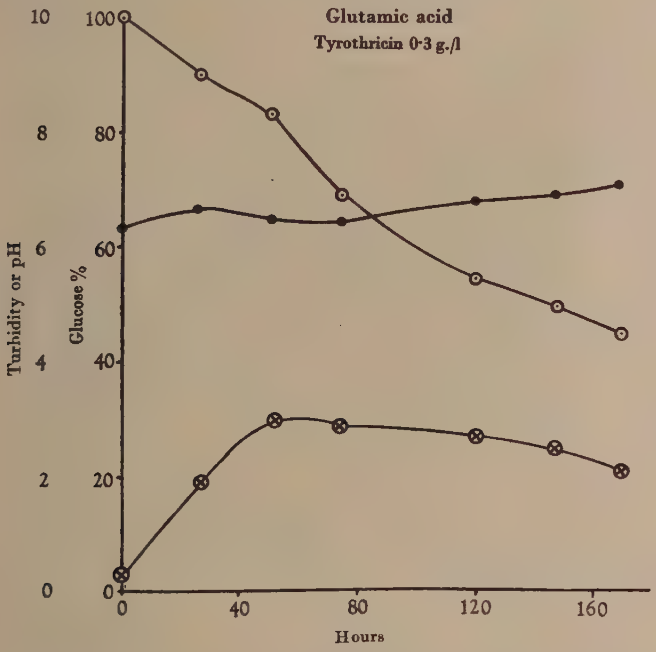

Fig. 1

Figs. 1–3. Metabolism of *B. brevis* in synthetic media with the stated nitrogen sources. Glucose utilization (%), −⊙——⊙−; ammonia utilization (%), --⊙-----⊙--; pH, −●——●−; turbidity (Spekker units—1 cm. cell), −⊗——⊗−; tyrothricin content (g./l.), --●-----●--. Final tyrothricin yield as stated.

It is not clear why, of the range of salts tested, ammonium succinate alone should be specific for tyrothricin production, nor why it should permit of higher yields than are obtained with amino-acids as nitrogen sources. In Figs. 1–3 the course of the fermentations in glutamic acid, ammonium citrate, and ammonium succinate media is shown diagrammatically. Ammonia values are not given for glutamic acid since the free ammonia content remained remarkably low and constant throughout the fermentation, indicating either an absence of deamination or immediate and complete utilization of the ammonia liberated by deamination.

The behaviour of the organism in the glutamic acid medium conforms with that outlined by Stokes & Woodward (1943). The ammonium citrate and ammonium succinate media give virtually identical metabolic pictures except that tyrothricin was produced only in the succinate medium. In both cases

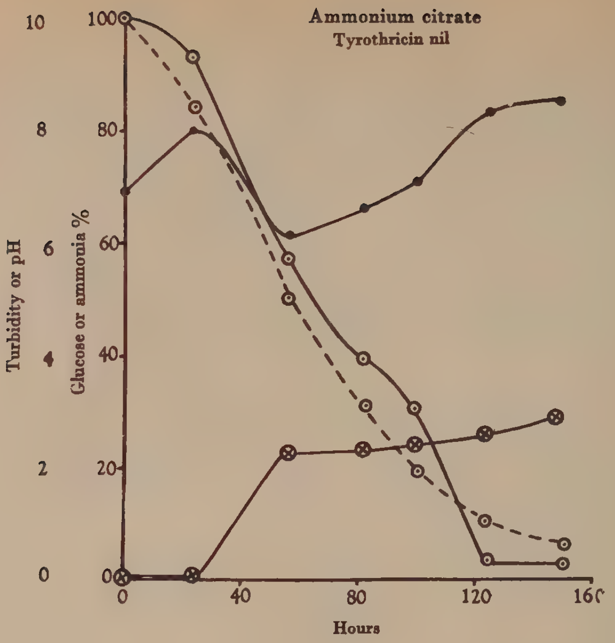
Ammonium citrate
Tyrothricin nil
Turbidity or pH
10
8
6
4
2
0
Glucose or ammonia %
100
80
60
40
20
0
0
40
80
120
160
Hours

Fig. 2

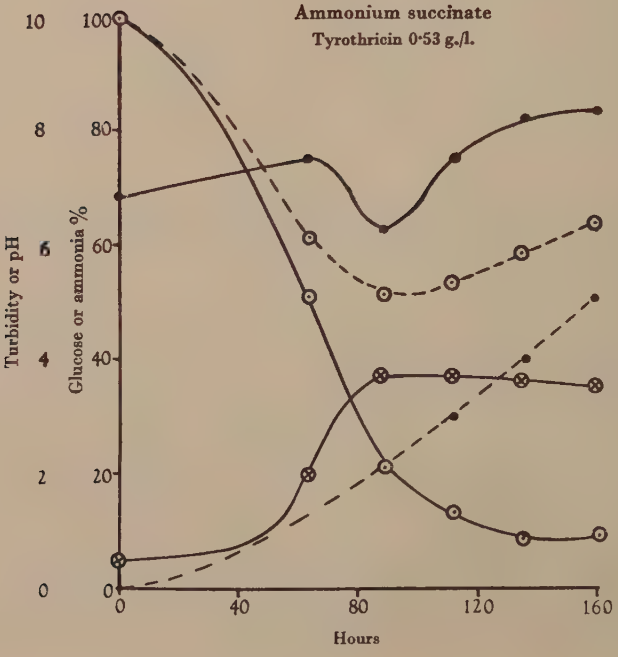
Ammonium succinate
Tyrothricin 0·53 g./l.
Turbidity or pH
10
8
6
4
2
0
Glucose or ammonia %
100
80
60
40
20
0
0
40
80
120
160
Hours

Fig. 3

an initial rise of pH was followed by a marked drop with a final rise usually to at least pH 8·5. Duplicate experiments rarely give identical curves, the degree of rise and fall of pH and the time at which the changes occur varying somewhat; but the general form of the changes was always as described. In the ammonium succinate medium variation of the initial pH between 5·8 and 7·5 had no effect on the final yield of tyrothricin, on the rate of use of nutrients, or on the rate of growth of the organism. The only feature calling for comment is that the higher the initial pH, the less marked was the first rise, and this perhaps would be expected.

The glucose utilization curves generally show a brief initial lag which, owing to loss of water by evaporation, may show up as an initial increase of the sugar concentration. Thereafter, utilization is rapid and almost complete by 120 hr., but the curve then flattens with some 5–10% of the initial sugar remaining unused. The phase of rapid glucose utilization is generally coincident with the fall of pH and, from several experiments with ammonium succinate, it appears that this pH fall is more extreme in runs showing the most rapid glucose metabolism. The final pH rise generally begins when carbohydrate decomposition is approaching completion, suggesting that acid accumulates during the active growth phase.

Ammonia utilization, in the case of the ammonium citrate medium, appears to follow that of glucose very closely. In the ammonium succinate medium, however, two distinct types of ammonia metabolism occur: (i) ammonia disappearance parallels that of glucose throughout; (ii) after about half the ammonia has been used (80 hr.), the ammonia concentration rises again (Fig. 3): this type is the more frequent. Such differences are found even in simultaneous replicate experiments, and do not appear to be correlated with special changes of pH, glucose metabolism or growth rate; the yield of tyrothricin is not affected. It was noted that, in most experiments, type (i) was accompanied by poor sporing, while in type (ii) sporing began early and became almost complete. However, this was not always the case, so it is not possible to conclude that the secondary formation of ammonia is the result of autolysis.

The turbidity data for both citrate and succinate media show an initial lag phase lasting for some 40 hr., followed by a similar period of rapid growth; after 80 hr. turbidity remains unchanged or falls slightly. In general, maximum turbidity coincides with the beginning of sporulation and occurs well before the various nutrients are exhausted.

The rate of tyrothricin formation in ammonium succinate media is also shown in Fig. 3. The final yield is not improved by further incubation.

Effect of addition of vitamin B-complex

It was shown by Appleby *et al.* (1947) that the addition of yeast extract to peptone medium markedly increased the yield of tyrothricin obtained in surface culture. In submerged culture (glutamic acid medium) the growth of *B. brevis* is stimulated by yeast extract, but the formation of tyrothricin is inhibited (see p. 146). Since it seems probable that the stimulation of growth is due to the presence of B-vitamins in the extract, whereas the inhibition of tyrothricin formation is due to the presence of proteins or their degradation products, it was decided to test the result of adding a product containing the members of the vitamin B-complex but free from proteins, amino-acids, etc. The commercial product 'B-Plex' (John Wyeth and Bro. Ltd., 25 Oldhill Place, London. N. 16) met these requirements. It is a fortified extract of rice bran containing (μg./ml.): aneurin hydrochloride 125, riboflavin 250, nicotinic acid 1250, pyridoxin hydrochloride 125, calcium pantothenate 625, choline 3000, inositol 1800, and biotin 1.

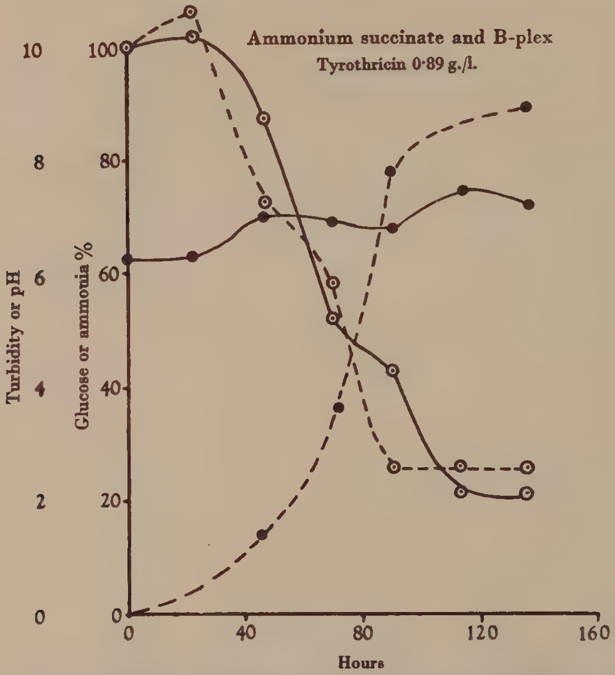

Fig. 4. Metabolism of *B. brevis* in ammonium succinate medium plus 0·5 % B-Plex. Symbols as for Figs. 1–3.

The addition of 0·5% (v/v) of this product to the ammonium succinate medium stimulated growth of *B. brevis*, and also markedly increased the yield of tyrothricin, *c.* 0·9 g./l. culture medium being obtained. Comparison of Figs. 3 and 4 shows that, although the rate of utilization of nutrients is not markedly affected, both the rate of formation and the final yield of antibiotic are increased. It appears therefore that, in submerged (though not in surface) culture, the effect of B-vitamins in increasing tyrothricin production is only apparent if proteins and amino-acid mixtures are absent.

Pilot-plant scale runs with the succinate—B-Plex medium were carried out in a glass-lined 200 l. fermentation vessel designed for larger scale work on antibiotics (Gordon, Grenfell, Knowles, Legge, McAllister & White, 1947). In general, similar results were obtained except that the yields of tyrothricin were lower (0·3–0·4 g./l.), but were obtained in a shorter period (120 hr.). The general form of the results makes it clear that the findings in aspirators can be applied, with proper technical modifications, to larger scale working.

The question as to which of the vitamins causes these effects was next investigated in aspirator-scale experiments. The inoculum for these tests was 10 ml. from a 3-day surface culture on the basal ammonium succinate medium. In each fermentation a single vitamin was added in the amount given below, up to six experiments being conducted simultaneously in order to obtain results as comparable as possible; a control experiment without added vitamin was included in each set. A typical series of results is given in Table 1; the vitamin concentrations used were equivalent to those present in the experiments with B-Plex.

Table 1. *Effect of vitamins of the B-complex on tyrothricin production*

Basal medium (g./l.): ammonium succinate 5·0; glucose 10·0; KH_2PO_4 0·5; K_2HPO_4 0·5; $MgSO_4,7H_2O$ 0·2; NaCl 0·01; $FeSO_4,7H_2O$ 0·01; $CaCl_2$ 0·25.

Vitamin added	Amount (mg./l. medium)	Yield of tyrothricin (g./l. medium)
Aneurin hydrochloride	0·625	0·60
Nicotinic acid	6·250	0·55
Pyridoxin	0·625	0·60
Pantothenic acid	3·125	0·34
Choline hydrochloride	15·000	0·36
Riboflavin	1·250	0·43
Inositol	9·000	0·29
Biotin	0·005	0·88
None	—	0·53

The analytical data obtained with pyridoxin and biotin are illustrated in Figs. 5 and 6, and may be compared with those for unsupplemented ammonium succinate (Fig. 3). In general, for all the separate vitamin runs, the data conformed closely to those for ammonium succinate without added vitamins. Ammonia utilization is rapid and almost complete in the case of pantothenic acid, choline or inositol; but is of the incomplete type in the case of the other vitamins. As indicated previously, however, the occurrence of one or the other type of ammonia metabolism is of uncertain significance. Figs. 5 and 6 have been chosen because they illustrate a feature of particular interest. The addition of biotin, riboflavin or inositol (in the amounts stated in Table 1) consistently delays the initial stages of the fermentation, and little utilization of glucose or ammonia takes place for *c.* 80 hr. (Fig. 6). Growth, as indicated by turbidity, shows a similar lag. Thereafter, the rate of metabolism is faster than usual, and by 120 hr. these fermentations have reached the same stage as the controls or those with other single vitamins present.

Pyridoxin, aneurin, nicotinic acid, pantothenic acid and choline have little, if any, effect on the rate of metabolism or growth of the organism (e.g. pyri-

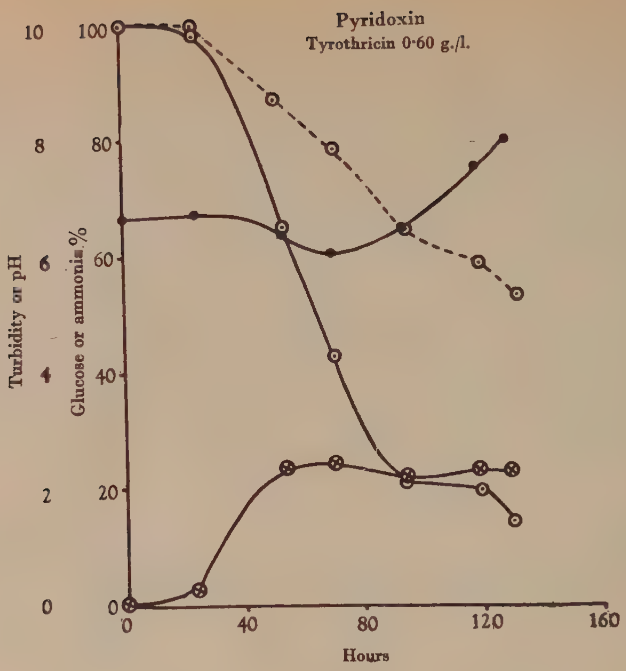

Fig. 5

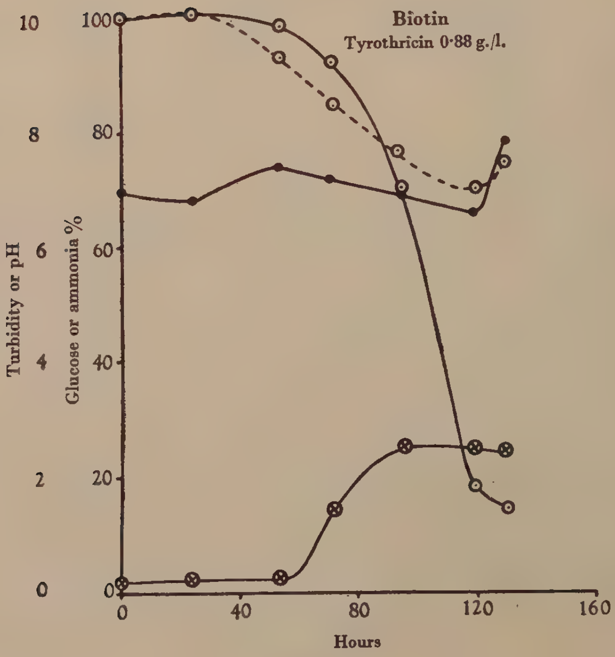

Fig. 6

Figs. 5, 6. Metabolism of *B. brevis* in ammonium succinate medium in the presence of pyridoxin and biotin. Symbols as for Figs. 1–3.

doxin, Fig. 5). It would seem, therefore, that the stimulating effect of B-Plex on the growth of *B. brevis* in the ammonium succinate medium is a composite one, and is not due to any single member of the vitamin B group. When all the vitamins are present, the initial delaying effect of biotin, riboflavin and inositol is not evident.

It is, however, clear from Table 1 and Fig. 6 that the stimulating effect of B-Plex on the tyrothricin yield is due to the presence of biotin, since addition of the latter alone (5 μg./l.) brings the yield of tyrothricin up to 0·88 g./l. medium as compared with the 0·89 g./l. obtained with B-Plex. There is evidence that aneurin and pyridoxin may aid in increasing the tyrothricin yield, as each give 0·60 g./l. as against controls of 0·53 g./l. The yield with nicotinic acid is unchanged, while riboflavin, pantothenic acid, choline and inositol all appear to decrease slightly the ultimate yield of tyrothricin or, alternatively, may merely delay its formation. It is clear from this that the stimulation or otherwise of the yield of tyrothricin cannot be predicted from the utilization of nutrients, and must be due to some more subtle effect on metabolism. Observation of the morphological appearance of the organism throughout each run cast no light on this point.

It is not proposed here to discuss further why biotin should have such a marked stimulating effect on the yield of tyrothricin, nor to relate this observation to previous work on the effect of vitamins on micro-organisms. The latter field has been fully reviewed recently by Peterson & Peterson (1945), but the only work with *B. brevis* appears to be that of Landy, Dicken, Bicking & Mitchell (1942), who found that biotin had a marked effect on metabolism. In view of the potential importance of this organism and the definite link between the action of biotin and the formation of tyrothricin, more detailed investigation of this relationship is desirable.

Urea as source of nitrogen

Investigations were also carried out with a synthetic medium in which the ammonium succinate was replaced by a corresponding amount of urea as the nitrogen source. The organism would not always grow satisfactorily on this medium, but when growth did occur it was accompanied by tyrothricin formation, the yields being *c.* 0·2 g./l. Fig. 7 shows that the metabolic picture again has its distinctive features. There is a lag phase of some 80 hr. before glucose and ammonia utilization commence properly, and little change of pH or turbidity occurs during this period. Thereafter utilization is rapid but falls off at higher concentrations than in the succinate medium, possibly because in the urea experiments the pH tends to fall to levels unfavourable to optimum growth and does not show the final steep rise which occurs in a really suitable medium.

Tyrothricin formation in peptone medium

After our strain F of *B. brevis* B.G. (Dubos) had been cultured for some 18 months, it suddenly developed the capacity to produce tyrothricin in deep culture in peptone media. This finding was unexpected, and resulted from a decision to investigate more closely the metabolism of the organism in the presence of peptone. Fig. 8 gives the analytical data for an experiment with the peptone-yeast extract medium described earlier. This experiment gave a tyrothricin yield of 0·20 g./l. medium. The results of a similar experiment in the same medium with the addition of glucose (10 g./l.) are given in Fig. 9; the tyrothricin yield was 0·15 g./l.

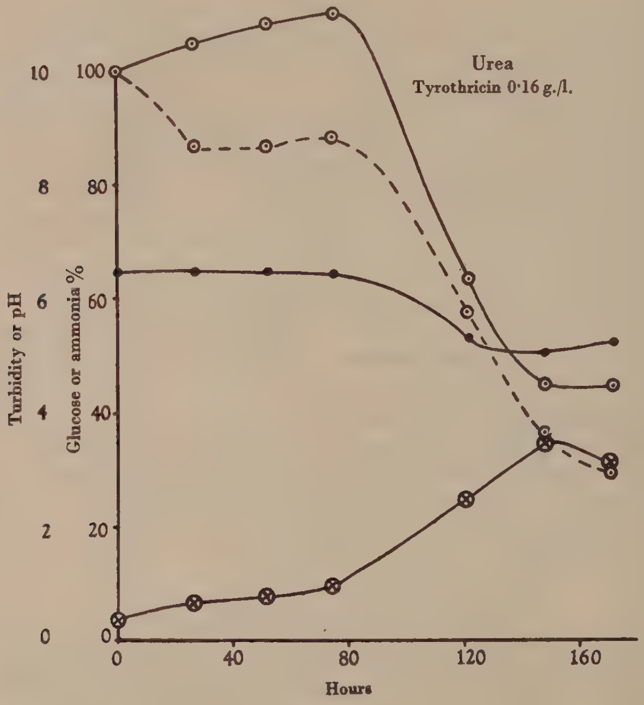

Fig. 7

Figs. 7–9. Metabolism of *B. brevis* in the presence of urea, peptone, and peptone + glucose, respectively. Symbols as for Figs. 1–3, except in Figs. 8 and 9 where ammonia is shown as mg./100 ml. instead of as percentage utilization.

Both in the presence and absence of glucose there is an initial liberation of ammonia into the medium, presumably as a result of proteolysis. In the medium without glucose this is particularly marked, and reaches a peak by 80 hr. Thereafter ammonia disappears at a correspondingly rapid pace, suggesting that the organism does not use ammonia initially in the presence of complex nitrogen sources, but does so later in the fermentation. This may be responsible for the low yield of tyrothricin obtained. To some extent the fall after 80 hr. may be due to loss of ammonia by aeration at a marked alkaline pH, but it seems improbable that this could account for the very rapid elimination shown in Fig. 8. The pH curve clearly reflects the initial accumulation and subsequent utilization of ammonia and is quite different from the pH curves for the synthetic media.

In the presence of glucose, the initial accumulation of ammonia is less marked and more prolonged. The fact that glucose is being metabolized is evidently responsible for this, since the organism is clearly using the sugar as a carbon source in preference to, or in addition to, the peptone. The extent of deamination is presumably less under these conditions, and the peak liberation of ammonia is correspondingly lower. Utilization of ammonia is again not marked until the later stages of the fermentation. The pH changes are less easy to interpret but seem to indicate an initial production of acid which outweighs ammonia liberation, followed by a rise of pH which may be due to metabolism of the acidic carbohydrate breakdown products once the initial attack on the glucose molecule is complete. These results support the view of Appleby *et al.* (1947) that *B. brevis* cannot be regarded as an organism incapable

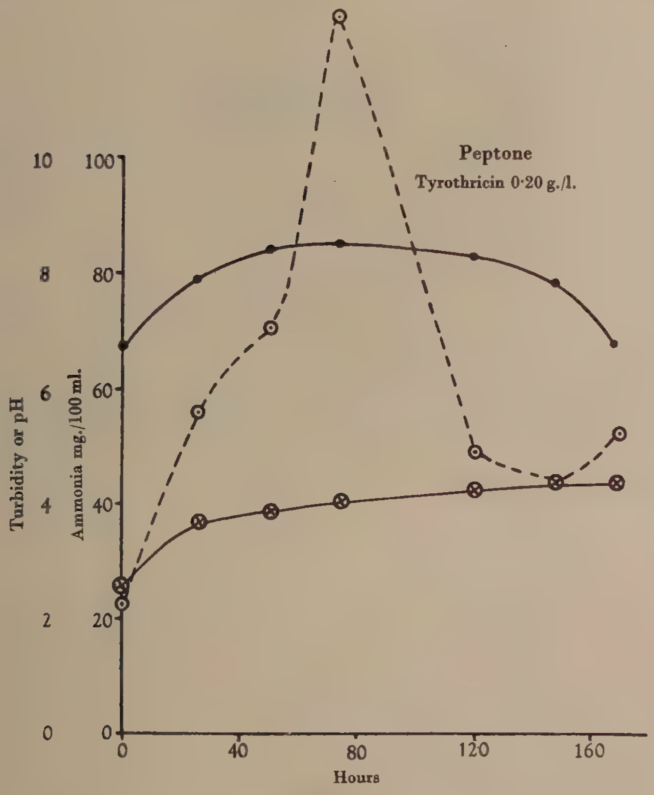

Fig. 8

of producing acid from carbohydrates. In all these experiments, the turbidity data do not indicate the initial growth lag found with synthetic media; the initial rate of growth is rapid and slows down to a steady value after *c.* 50 hr.

Despite the differences caused by the presence or absence of glucose, it is clear that in the presence of peptone the metabolism of submerged cultures differs radically from that in synthetic media. In fact, the data suggest that when peptone is present the primary consideration of the organism is its source of carbon rather than of nitrogen. In the synthetic media both appear to call forth an equal degree of metabolic activity, and this may constitute a clue to the fact that normally *B. brevis* grows well but does not form tyrothricin (or does so only to a minor degree) when peptone is present. Even in the above experiments the yield of tyrothricin is only one-quarter of that obtainable in surface culture on the same media in half the time. It is not proposed to discuss the reasons why *B. brevis* should suddenly develop the capacity of producing tyrothricin in submerged culture in the presence of peptone after earlier experiments had failed to demonstrate any such ability. Lewis, Dimick & Feustel (1945) have also reported briefly the formation of tyrothricin in the presence of peptone in the case of a particular substock of the Dubos B.G. strain which had been maintained as a pure culture for some time.

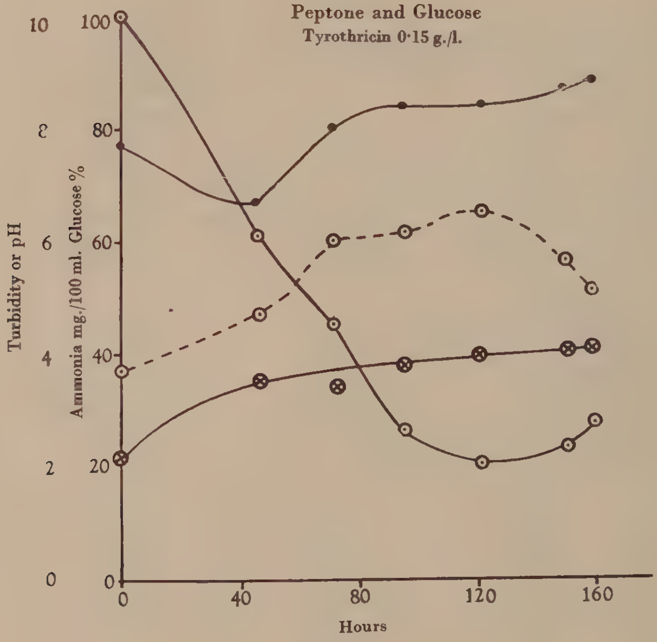

Fig. 9

The authors wish to express their gratitude to Mr A. J. C. Gormley, Managing Director of John Wyeth and Bro. Ltd., for permission to publish the above results.

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Production of Antifungal and Antibacterial Substances by Fungi; Preliminary Examination of 166 Strains of Fungi Imperfecti

By P. W. BRIAN and H. G. HEMMING

Imperial Chemical Industries Ltd., Butterwick Research Laboratories, Welwyn, Herts

SUMMARY: One hundred and sixty-six strains of Fungi Imperfecti have been examined primarily for the production of antifungal substances, though antibacterial substances have also been sought in most cases.

Antifungal substances were produced by several species of *Aspergillus* and *Penicillium*, *Fusarium caeruleum*, *Metarrhizium glutinosum*, *Stachybotrys atra*, *Trichoderma viride*, *Gliocladium catenulatum* and *Trichothecium roseum*. Antibacterial substances were produced by species of *Aspergillus*, *Fusarium*, *Penicillium*, *Stachybotrys* and *Trichoderma*.

The chemical nature of the substances responsible for this activity is known or can be suggested in some cases, but others are worthy of further investigation.

Several surveys of the production of antibacterial substances by fungi have been published recently (Wilkins & Harris, 1942, 1943, 1944 *a*, *b*, *c*; Wilkins, 1945, 1946; Robbins, Hervey, Davidson, Ma & Robbins, 1945), from which it appears that many of the Ascomycetes, Basidiomycetes and Fungi Imperfecti show marked antibacterial activity but few, if any, of the Phycomycetes. No comparable surveys of the production of antifungal substances have been published, though many substances originally studied for their antibacterial activity have since been found to be also toxic to fungi. The present account deals with a number of Fungi Imperfecti examined primarily for antifungal activity; a preliminary examination for antibacterial activity has also been made in most cases. Species of *Penicillium*, *Aspergillus* and *Trichoderma* account for a high proportion of the strains examined, but representatives of seventeen other form-genera have been included. Further studies of certain of the strains found to produce antifungal substances have resulted in the isolation in pure form of the antibiotics viridin, glutinosin, gladiolic acid and 'curling-factor'; the relevant references to this work are given in a later section of this paper.

EXPERIMENTAL MATERIALS AND METHODS

The fungi examined were obtained from various sources. Many were isolated in this laboratory from soils or other materials, and many were obtained from the National Collection of Type Cultures. The writers are also indebted to Dr G. R. Bisby for cultures of *Trichoderma*, *Stachybotrys* and *Memnoniella* and to Mr George Smith for cultures of *Trichoderma* and *Gliocladium*. All identifications have been checked as far as possible and a number of the more interesting Penicillia (*P. terlikowskii* and members of the *P. nigricans*-

janczewskii series) have been submitted for identification to Dr K. B. Raper of the United States Department of Agriculture.

The antifungal and antibacterial properties of these fungi have been examined in most cases by two methods. The first and most simple method was by observation of antagonisms in agar-plate cultures. The second method involved growing the fungi on liquid media with periodical assays of the culture filtrates for antifungal and antibacterial activity. These methods are described in greater detail below.

Antagonism in agar culture

The agar medium used had the following composition: glucose 10 g.; peptone (Evans Medical Supplies, London) 10 g.; Lab-Lemco meat extract 3 g.; sodium chloride 5 g.; agar 15 g.; distilled water 1000 ml. Plates of this medium were inoculated with a transverse streak of spores of the fungus to be examined, or by a mycelial transplant if spores were not sufficiently abundantly produced. The inoculated plates were incubated for 4 days at 25°. Using a platinum loop, 24 hr. broth cultures of two bacteria (*Staphylococcus aureus* and *Salmonella typhi*) and a suspension in sterile broth of cell material of the animal-pathogenic fungus *Endomycopsis albicans* (Vuill.) Dekker, were then streaked at right angles to the advancing edge of the fungus colony. The plates were incubated for a further 24–28 hr. at 37° after which they were examined and the degree of antagonism, as represented by the presence or absence, adjacent to the fungus colony, of a clear zone where no growth of the test organism had taken place, recorded as follows:

− = no indication of antagonism,
+ = zone of inhibition not more than 0·5 cm.,
++ = zone of inhibition greater than 0·5 cm.,
(+) = an apparent diminution in vigour of growth of the test organism without production of a clear zone, observed in some tests.

The liquid culture method

The mould to be examined was grown on 30 ml. lots of medium dispensed in 100 ml. Pyrex flasks. In some of the earlier experiments 15 ml. of medium in 9 cm. Petri dishes was used (cf. Weindling, 1934), but this method was discarded because of the prevalence of contaminations and difficulty of handling. In general 20–25 flasks of each medium used were inoculated, incubated at 25°, and five were withdrawn at random and bulked for assay. Samples were taken in this manner usually after 6, 8, 10 and 12 days' incubation; with some more rapidly growing forms (e.g. *Trichoderma*) sampling was begun earlier, and was carried on longer (up to 21 days) with the more slowly growing organisms. The liquid medium was filtered off and assayed for fungistatic activity by the *Botrytis allii* spore-germination technique (Brian & Hemming, 1945) and for antibacterial activity by the usual serial dilution technique, using *Staphylococcus aureus* as test organism, growth being estimated turbidimetrically.

The media used in this work have been varied from time to time as experience was gained and bearing in mind, in some cases, known preferences of some of the organisms. The media used were Weindling, Weindling N, Czapek-Dox, Raulin-Thom, Cornsteep and Barham & Smits. Of these, the composition of Weindling, Czapek-Dox, Raulin-Thom and Cornsteep have been previously given (Brian, Curtis & Hemming, 1946). Weindling N is a variant of the standard Weindling medium with the same amount of nitrogen supplied as sodium nitrate instead of as ammonium tartrate. The Barham & Smits medium is one with high sugar content recommended (Barham & Smits, 1936) for production of kojic acid by *Aspergillus flavus*.

RESULTS

The results are summarized in Table 1. To economize in space the results of all assays have not been given but only the highest assay observed at any of the samplings. The activities of culture filtrates are indicated as follows:

2 signifies inhibition of spore germination or bacterial growth at a dilution of 1 in 4 (1 in 2^2) but not at 1 in 8,

3 signifies inhibition of spore germination or bacterial growth at a dilution of 1 in 8 (1 in 2^3) but not at 1 in 16,

and so on accordingly. In the case of the *B. allii* spore-germination test the least dilution examined was 1 in 2; activity at this level but not at 1 in 4 was recorded as activity 1, no activity at 1 in 2 was recorded as 0. In the case of the antibacterial tests the results of the first dilution were ignored on all media and of the first two dilutions with Raulin-Thom and Weindling, as the culture medium alone was found to inhibit growth on occasion; activity 0 represents, therefore, no activity at a dilution of 1 in 8 with culture filtrates from Raulin-Thom and Weindling, no activity at 1 in 4 for all other media.

Detailed consideration of results

The fungi examined can most conveniently be discussed in groups.

Aspergillus. Only three strains of *Aspergillus* of the 22 examined showed any marked activity when grown on liquid media, though several more were active in agar culture. A strain of *A. clavatus* (no. 88) showed both antifungal and antibacterial activity. Strains of this species have been shown (Katzman, Hayes, Cain, van Wyk, Reithel, Thayer, Doisy, Gaby, Carroll, Muir, Jones & Wade, 1944; Waksman, Horning & Spencer, 1943) to produce the antibiotic substance known as clavacin or patulin (anhydro-3-hydroxymethylene-tetrahydro-1:4-pyrone-2-carboxylic acid) and it is the production of this substance which probably accounts for the effects observed.

Two strains of *A. terreus* (nos. 278 and 279) possess marked antifungal and antibacterial activity. This species is biochemically very versatile and substances isolated from its culture filtrates include clavacin (Kent & Heatley, 1945), citrinin (Timonin, 1942; Thom & Raper, 1945) and itaconic acid (Calam, Oxford & Raistrick, 1939), all of which are known to possess antifungal and antibacterial properties.

Fusarium. *F. caeruleum* apparently produces an antifungal substance, though subsequent experiments have given conflicting results. Three unidentified *Fusarium* spp. (nos. 37, 42 and 47) produce an antibacterial substance. Little work has been reported on the isolation of antibiotics produced by *Fusarium* spp., though Arnstein, Cook & Lacey (1946) have recently reported the isolation of an antibacterial pigment—javanicin ($C_{15}H_{14}O_6$)—from culture filtrates from liquid culture of *F. javanicum*.

Metarrhizium. *M. glutinosum* showed marked antifungal activity but no antibacterial activity. The isolation of an antifungal substance, glutinosin ($C_{42}H_{60}O_{16}$), from culture filtrates, has already been reported (Brian & McGowan, 1946).

Penicillium. The strains falling into the *P. chrysogenum* series (nos. 25, 26, 76, 238 and probably also 182 and 188) show marked antibacterial activity (to *Staphylococcus aureus* but not to *Salmonella typhi* in the agar culture test) but no antifungal activity. This is almost certainly due to penicillin production. The slight antibacterial activity shown by *Penicillium citrinum* (no. 54) is probably due to production of citrinin and the moderate antifungal and antibacterial activity of strains of *P. cyclopium* and *P. puberulum* to production of penicillic acid (γ-keto-β-methoxy-δ-methylene-Δ^{α}-hexenoic acid) (Alsberg & Black, 1913; Birkinshaw, Oxford & Raistrick, 1936).

Several strains of *P. expansum* showed antibacterial activity; Anslow, Raistrick & Smith (1943) have shown that strains of this mould produce clavacin (patulin). Both strains of *P. gladioli* examined were actively antifungal and antibacterial; Brian, Curtis, Grove, Hemming & McGowan (1946) have shown this to be due to production of gladiolic acid ($C_{11}H_{10}O_5$), which they have isolated in pure form. Six strains of *P. janczewskii* were examined, all of which save one had been isolated from Wareham Heath soil. Though the antifungal activity as expressed by suppression of spore germination was only moderate, they all produced extraordinary distortions of *Botrytis allii* hyphae. A pure substance ('curling-factor') responsible for this interesting physiological effect has been isolated (Brian *et al.* 1946; McGowan, 1946). Two related strains of the *P. nigricans-janczewskii* series produce antibacterial and antifungal culture filtrates, without the distorting effect; these are at present the subject of investigation.

The marked antifungal and antibacterial activity of *P. terlikowskii* has been shown (Brian, Hemming & McGowan, 1945; Brian, 1946) to be due to production of gliotoxin ($C_{13}H_{14}N_2S_2O_4$).

P. lilacinum is interesting in that it has shown marked antagonistic effects in agar culture but when grown in liquid culture the culture filtrates have shown little if any activity. In addition to those already mentioned a number of other *Penicillium* spp. have shown slight antifungal or antibacterial activity, but no further comment is at present possible.

Stachybotrys and Memnoniella. Of nine strains of *Stachybotrys atra* examined six show antifungal or antibacterial activity. The remaining six strains, and

Table 1. *Antifungal and antibacterial activity of* 166 *strains of Fungi Imperfecti*

Accession no.	Organism	Antifungal activity of culture filtrates (*Botrytis allii*)						Antibacterial activity of culture filtrates (*Staphylococcus aureus*)						Antagonistic effects in agar culture		
		WA	WN	CD	RT	CS	BS	WA	WN	CD	RT	CS	BS	*Staph.*	*Salm.*	*End.*
88	*Aspergillus clavatus* Desm.	0	0	.	.	.	3	0	4	.	.	.	6	++	++	−
268	*A. flavus* Link	.	.	.	.	.	0	.	.	.	.	.	4	−	−	−
277	*A. fumaricus* Wehmer (3892)	.	.	.	.	.	0	.	.	.	.	.	0	+	++	−
266	*A. oryzae* (Ahlb.) Cohn	.	.	.	.	.	0	.	.	.	.	.	0	+	−	−
274	*A. tamarii* Kita (5[illegible]9)	.	.	.	.	.	0	.	.	.	.	.	0	+	(+)	−
275	*A. tamarii* (1884)	.	.	.	.	.	0	.	.	.	.	.	0	+	−	+
276	*A. tamarii* (3910)	.	.	.	.	.	0	.	.	.	.	.	0	−	(+)	−
278	*A. terreus* Thom (981)	.	.	7	4	.	4	.	.	5	0	.	2	++	++	−
279	*A. terreus* (3911)	.	.	8	8	.	6	.	.	9	5	.	7	++	++	+
255	*Aspergillus* sp.	.	.	.	.	.	0	.	.	.	.	.	4	.	.	.
259	*Aspergillus* sp.	.	.	.	.	.	0	.	.	.	.	.	2	.	.	.
35	*Fusarium caeruleum* (Lib.) Sacc.	8	6	.	.	.	.	0	0	.	.	.	.	−	−	+
37	*Fusarium* sp.	0	0	.	.	.	.	4	0	.	.	.	.	−	−	−
42	*Fusarium* sp.	0	0	.	.	.	.	3	0	.	.	.	.	−	−	−
47	*Fusarium* sp.	0	0	.	.	.	.	3	0	.	.	.	.	−	−	−
224	*Gliocladium catenulatum* Gilman & Abbott (6597)	0	.	0	8	0	.	0	.	0	0	0	.	+	−	−
222	*G. fimbriatum* Gilman & Abbott (6599)	0	.	0	2	0	.	0	.	0	0	0	.	−	−	−
11	*Gliocladium* sp.	2	4	.	.	.	.	0	0	.	.	.	.	−	−	−
173	*Metarrhizium glutinosum* Pope	5	.	5	8	4	.	0	.	0	0	0	.	−	−	+
65	*Penicillium chrysogenum* Thom (589)	0	0	0	.	0	.	3	3	8	.	9	.	++	−	−
25	*P. chrysogenum* series	4	0	2	.	0	.	3	0	10	.	12	.	++	−	−
76	*P. chrysogenum* series	0	0	0	.	0	.	0	0	8	.	8	.	++	−	−
238	*P. chrysogenum* series	.	.	0	0	0	.	.	.	5	4	5	.	++	−	−
24	*P. citreo-viride* Biourge	0	2	.	.	.	.	0	0	.	.	.	.	−	−	−
54	*P. citrinum* Thom (3539)	0	0	.	.	.	.	0	3	.	.	.	.	+	+	−
50	*P. cyclopium* Westling	0	0	.	.	.	.	0	5	.	.	.	.	−	−	−
160	*P. cyclopium*	2	.	1	2	0	.	0	.	3	4	0	.	+	+	−
161	*P. cyclopium*	0	.	2	2	0	.	0	.	0	0	0	.	(+)	−	−
33	*P. digitatum* Sacc.	0	0	0	0	0	.	0	3	3	0	0	.	+	+	−
57	*P. expansum* Link (593)	0	0	.	.	.	.	3	5	.	.	.	.	+	++	−
102	*P. expansum*	0	0	.	.	.	.	2	3	.	.	.	.	++	++	−
104	*P. expansum*	0	.	.	.	.	.	0	.	.	.	.	.	++	++	−
105	*P. expansum*	0	.	.	.	.	.	0	.	.	.	.	.	++	++	−
233	*P. flavidorsum* Biourge	0	.	3	3	2	.	0	.	0	0	0	.	−	−	−
242	*P. gilmanii* Thom	0	.	0	0	0	.	0	.	0	0	0	.	−	−	+
251	*P. gilmanii*	0	.	0	0	0	.	0	.	0	0	0	.	−	−	(+)
59	*P. gladioli* McCull. & Thom (3994)	4	2	5	6	0	.	4	3	4	3	0	.	++	++	+
206	*P. gladioli*	4	.	4	5	1	.	0	.	3	3	0	.	++	++	+
29	*P. janczewskii* Zal.	2	.	0	.	.	.	0	5	.	.	.	.	−	−	−
240	*P. janczewskii*	1	.	2	3	3	.	0	.	0	0	0	.	−	−	(+)
246	*P. janczewskii*	0	.	1	2	2	.	0	.	0	0	0	.	−	−	(+)
249	*P. janczewskii*	3	.	2	3	2	.	0	.	0	0	0	.	−	−	(+)
250	*P. janczewskii*	2	.	2	2	0	.	0	.	0	0	0	.	−	−	(+)
252	*P. janczewskii*	2	.	3	3	1	.	0	.	0	0	0	.	−	−	(+)
227	*P. jenseni* Zal.	0	.	0	0	0	.	0	.	0	0	0	.	−	−	(+)
60	*P. lilacinum* Thom (584)	2	0	0	0	0	.	0	0	0	0	0	.	++	−	++
235	*P. lilacinum*	0	.	0	0	0		0	.	0	0	0	.	+	−	(+)
243	*P. nigricans-janczewskii* series	5	.	6	6	2	.	3	.	3	3	0	.	−	−	−
248	*P. nigricans-janczewskii* series	5	.	5	5	4	.	0	.	0	3	0	.	−	−	−
26	*P. notatum* Westling (4222)	2	2	0	2	0	.	4	4	7	3	7	.	++	+	−
66	*P. oxalicum* Currie & Thom (983)	2	0	.	.	.	.	0	0	.	.	.	.	−	−	−
247	*P. puberulum* Bain.	2	.	0	2	0	.	0	.	0	0	2	.	−	−	+
63	*P. roquefortii* Thom (528)	2	0	.	.	.	.	5	5	.	.	.	.	−	−	−
241	*P. terlikowskii* Zal.	7	.	5	6	5	.	5	.	6	5	6	.	++	++	++
244	*P. terlikowskii*	4	.	3	4	5	.	4	.	2	3	4	.	++	++	++
295	*P. terlikowskii*	2	.	2	4	2	.	4	.	3	3	3	.	++	++	++
79	*Penicillium* sp.	0	2	.	.	.	.	0	0	.	.	.	.	−	−	−
91	*Penicillium* sp.	2	2	.	.	.	.	0	0	.	.	.	.	+	+	−
103	*Penicillium* sp.	2	2	.	.	.	.	0	0	.	.	.	.	−	−	−
107	*Penicillium* sp.	2	0	.	.	.	.	.	.	.	.	.	.	−	−	−
110	*Penicillium* sp.	2	.	.	.	.	.	.	.	.	.	.	.	(+)	−	−

111	*Penicillium* sp.	0	1	.	.	.	.	.	.	.	.	.	.	−	−	−
112	*Penicillium* sp.	0	0	.	.	.	.	.	.	.	.	.	.	+	−	−
182	*Penicillium* sp.	0	.	.	.	.	.	.	.	.	.	.	.	++	++	−
188	*Penicillium* sp.	0	.	.	.	.	.	.	.	.	.	.	.	++	−	−
194	*Penicillium* sp.	2	.	.	.	.	.	.	.	.	.	.	.	+	++	−
217	*Penicillium* sp.	0	.	.	.	.	.	.	.	.	.	.	.	++	++	−
228	*Penicillium* sp.	3	.	.	.	.	.	.	.	.	.	.	.	−	−	−
55	*Scopulariopsis brevicaule* (Sacc.) Bain. (580)	2	2	.	.	.	.	0	0	.	.	.	.	−	−	−
223	*Stachybotrys atra* Corda (6436)	2	.	6	0	2	.	0	.	0	0	0	.	−	−	++
280	*S. atra*	.	.	0	.	.	.	.	.	.	.	.	.	+	−	+
281	*S. atra*	.	.	3	.	.	.	.	.	.	.	.	.	++	−	+
283	*S. atra*	.	.	0	.	.	.	.	.	.	.	.	.	++	−	−
284	*S. atra*	.	.	4	.	.	.	.	.	.	.	.	.	+	−	+
285	*S. atra*	.	.	0	.	.	.	.	.	.	.	.	.	+	−	+
41	*Trichoderma koningi* Oud. (3676) (= *T. viride*)	4	.	6	2	.	.	0	.	0	0	.	.	−	++	++
2	*T. viride* Pers. ex Fries	10	.	6	.	.	.	.	.	.	.	.	.	−	−	−
3	*T. viride*	12	.	10	8	.	.	0	.	0	0	.	.	+	++	+
5	*T. viride*	0	.	0	.	.	.	.	.	.	.	.	.	+	++	+
6	*T. viride*	8	.	2	.	.	.	.	.	.	.	.	.	−	−	−
7	*T. viride*	2	.	0	0	0	.	0	.	0	0	0	.	++	−	−
9	*T. viride*	2	.	2	.	.	.	.	.	.	.	.	.	−	−	−
10	*T. viride*	12	.	10	11	.	.	0	.	0	0	.	.	−	−	(+)
12	*T. viride*	8	.	6	.	.	.	6	.	4	.	.	.	+	+	+
121	*T. viride*	0	0	.	.	.	.	0	0	.	.	.	.	+	++	−
123	*T. viride*	2	2	.	.	.	.	0	0	.	.	.	.	−	−	−
124	*T. viride* (ex *Hypocrea rufa*)	0	0	.	.	.	.	0	0	.	.	.	.	+	++	−
126	*T. viride*	0	0	.	.	.	.	0	0	.	.	.	.	+	++	+
138	*T. viride*	0	0	.	.	.	.	0	0	.	.	.	.	+	++	−
145	*T. viride*	0	0	.	.	.	.	0	0	.	.	.	.	+	++	−
148	*T. viride*	2	2	.	.	.	.	0	0	.	.	.	.	−	−	−
150	*T. viride*	4	0	.	.	.	.	0	0	.	.	.	.	+	+	−
151	*T. viride*	2	0	.	.	.	.	0	0	.	.	.	.	+	+	−
153	*T. viride*	2	2	.	.	.	.	0	0	.	.	.	.	++	++	−
154	*T. viride*	2	2	.	.	.	.	0	0	.	.	.	.	++	++	−
155	*T. viride*	0	0	.	.	.	.	0	0	.	.	.	.	++	++	−
187	*T. viride*	3	.	1	2	.	.	0	.	10	0	.	.	(+)	++	(+)
207	*T. viride*	6	.	5	0	.	.	3	.	3	3	.	.	++	++	++
208	*T. viride*	5	.	6	3	.	.	0	.	0	6	.	.	++	++	+
211	*T. viride*	10	.	2	11	6	.	5	.	2	6	3	.	++	++	+
213	*T. viride*	13	.	8	15	.	.	0	.	0	0	.	.	−	−	+
214	*T. viride*	9	.	8	7	6	.	0	.	0	0	0	.	−	−	+
218	*T. viride*	12	.	7	14	.	.	0	.	0	0	.	.	−	−	−
232	*T. viride*	0	.	0	0	.	.	0	.	0	0	.	.	++	++	++
39	*Trichothecium roseum* Link	6	6	.	.	.	.	0	0	.	.	.	.	−	−	−

Abbreviations for media:

WA = Weindling
WN = Weindling N
CD = Czapek-Dox
RT = Raulin-Thom
CS = Cornsteep
BS = Barham & Smits

Abbreviations for test organisms: *Staph.* = *Staphylococcus aureus*; *Salm.* = *Salmonella typhi*; *End.* = *Endomycopsis albicans*. The numbers given after the names of some of the fungi are their numbers in the National Collection of Type Cultures. For explanation of numbers and symbols see text.

The following strains showed no antifungal or antibacterial activity: Accession no. 157, *Alternaria solani* Jones & Grout (2925); 159, *Alternaria* sp. (1007); 270, 271 (596), 272, 273, *Aspergillus flavus* Link; 229, *A. glaucus* series; 32, *A. niger* van Tiegh.; 265 (598), 267, *A. oryzae* (Ahlb.) Cohn; 86, 97, 253, *A. sydowi* (Bain. & Sart.) Thom & Church; 257, *A. versicolor* (Vuill.) Tirabosch.; 45, *Botryosporium* sp.; 82, *Botrytis cinerea* Fr.; 93, *Cladosporium fulvum* Cooke; 31, *C. herbarum* Fr.; 94, *Colletotrichum lini* Bolley; 156, *Eidamia catenulata* Horne & Williamson; 16, *Fusarium avenaceum* (Fr.) Sacc.; 14, *F. culmorum* (W.G.Sm.) Sacc.; 15, *F. fructigenum* Fr. (3097); 48, 49, *Fusarium* sp.; 53 (1730), 230, *Gliocladium roseum* Bain.; 23, *Macrosporium sarcinaeforme* Cav.; 288, 289, 290, *Memnoniella echinata* (Riv.) Galloway; 52, *Paecilomyces varioti* Bain. (3089); 69, *Penicillium commune* Thom (3974); 78, *P. cyclopium* Westl.; 71, *P. luteum* Zukal. (585); 70, *P. pinophilum* Hedgcock (1151); 72, *P. pfefferianum* (Wehm.) Westl. (3936); 51, *P. purpurogenum* Stoll (586); 68, *P. rugulosum* Thom (592); 226, *P. spinulosum* Thom; 77, *P. tardum* Thom; 67, *P. terrestre* Jensen (763); 61, *P. viridicatum* Westl. (3998); 83, 95, 106, *Penicillium* sp.; 158, *Phoma alternariacearum* Brooks & Searle (1135); 114, *Pullularia nigra* Berkh. (1745); 115, *P. pullulans* Berkh. (1719); 282, 286, 287, *Stachybotrys atra* Corda; 62 (3883), 1, 4, 8, 27, 28, 122, 125, 135, 136, 146, 147, 149, 152, 156, *Trichoderma viride* Fr.; 74, *Verticillium alboatrum* Reinke & Berth.; 118, *Verticillium* sp.

three strains of *Memnoniella echinata*, included because of the possible identity of this form with *Stachybotrys atra* (Bisby, 1943, 1945; Zuck, 1946), showed neither type of activity. *S. atra* is at present being studied in greater detail.

Trichoderma and Gliocladium. Three well-defined groups can be seen in *Trichoderma*:

Group 1: showing marked antifungal activity but no antibacterial activity. This includes strains 2, 3, 10, 213, 214 and 218.

Group 2: showing both antifungal and antibacterial activity. This includes strains 12, 207, 208, 211.

Group 3: showing no activity, either on agar or in liquid culture. This, the largest group, includes strains 1, 4, 8, 27, 28, 62, 122, 125, 135, 136, 146, 147. 149, 152 and 156.

The strains in group 1 are all of the pigmented type; this type of activity is probably due to the production of viridin (Brian & McGowan, 1945; Brian, Hemming, Curtis & McGowan, 1946). Strains 3, 10, 213 and 214 are known to produce viridin. The strains in group 2 are all non-pigmented and probably all produce gliotoxin, which is both antibacterial and antifungal. Strains 12, 207 and 211 are known to produce this substance (Brian, 1944; Brian & Hemming, 1945).

In addition to these well-defined groups there are many strains which show various types of activity in agar culture but none when grown on liquid media. This effect is also to be noticed with fungi from other genera. One strain in particular is worth noting, no. 7, which appears to produce a substance active against *Staphylococcus* but not against *Salmonella* or the test fungi, suggesting that viridin and gliotoxin may not be the only antibiotic substances produced by *Trichoderma viride*.

The probable identity of Weindling's '*Gliocladium fimbriatum*', from which gliotoxin was first isolated, with *Trichoderma viride*, has been discussed elsewhere (Brian, 1944). It is of interest here to note that an authentic *Gliocladium fimbriatum* shows very slight antifungal activity, but that *G. catenulatum* is distinctly more active in this respect and is worth further investigation.

Other fungi. Fungi from other form-genera have all given negative results with the exception of *Trichothecium roseum* which produced antifungal culture filtrates. The antagonistic properties of this organism have been previously reported by Greaney & Machacek (1935).

General observations

As previously mentioned this survey has been carried out primarily to obtain information on the production of antifungal substances by moulds. Approximately one-third of the strains examined show antifungal activity. This proportion is weighted by the fact that where a species showed promise considerable numbers of strains were examined (especially in *Trichoderma*). After making due allowance for this, however, it will be seen that quite a high proportion of Fungi Imperfecti produce substances toxic to another mould (*Botrytis allii*).

Two limitations in the technique must also be borne in mind when considering the general implication of these results. First, only one test fungus has been used in the liquid culture experiments—*Botrytis allii*. It is possible that a completely different picture might have been obtained if other test fungi had been used. No definite judgement on this possibility can be made at present but the antifungal metabolic products which have been isolated in these laboratories (viridin, glutinosin, gladiolic acid and 'curling-factor') and others are generally active against most fungi other than the one specifically producing them. This technical limitation, then, may not be of great importance.

A more important limitation undoubtedly is that the spore-germination tests have all been carried out at pH 3·5 and acid-sensitive antifungal materials might well be destroyed under those conditions. The numerous recorded discrepancies between the agar culture method (carried out at pH 6·5–7) and the liquid culture method may be due to this. In future surveys it is proposed to carry out assays at pH 3·5 and at pH 6·5 or 7·0 so that both acid-sensitive substances and those stable only in acid solutions (e.g. viridin) can be detected.

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A Simple Plate Method for Multiple Tests of the Antibacterial Activity of many Bacteria against other Bacterial Strains

By N. G. HEATLEY
The Sir William Dunn School of Pathology, University of Oxford

SUMMARY: The organisms whose antibacterial powers are to be investigated are grown in parallel streaks on cellophan superposed on an agar plate. The cellophan is stripped off and the test organisms are streaked on the sterile agar surface thereby revealed, at right angles to the first streaks. After incubation, growth of the test organisms will be interrupted in those areas in which an inhibitor has diffused through the cellophan from the first organisms. There are some limitations in the application of the method.

During some recent work (Heatley & Florey, 1946) a strain of *Bact. coli* (*Escherichia coli*) was found to produce an antibiotic which inhibited some coliform organisms at a very high dilution, other coliform organisms being unaffected. Examination of a number of other strains of *Bact. coli* (M. A. Jennings, unpublished) showed that a few of them also formed antibiotics which were highly specific in the same way, inhibiting some strains of coliforms but not others, but differing from each other and from the first inhibitor examined in regard to the particular strains against which they were active.

The fact that the usual methods of detecting antibiotic action, employing only a few different strains, revealed a number of different highly specific inhibitors, suggested that there might be inhibitors formed by other strains of *Bact. coli* which had escaped detection because the test organisms used were not susceptible.

A simple test was required by which several strains of *Bact. coli* could be tested for antibacterial activity against several different test organisms. The method described below, which embodies more or less well-known principles (e.g. Gratia, 1944), was found to work satisfactorily, and may have wider applications, not only for the detection of antibiotic activity or the comparison of range of antibacterial action, but perhaps also for the selection of strains of bacteria which have special nutritional requirements, or of those which form an excess of certain growth factors, etc.

Principle of the method

The surface of a rather deep nutrient agar plate is covered with a circular sheet of cellophan and is then dried. The organisms to be tested are sown in parallel streaks on the surface of the cellophan (primary streaks), and the plate is incubated for 16–20 hr. (Pl. 1 A). The cellophan, together with the organisms growing on it, is stripped off, and the test organisms (secondary streaks) are then sown across the surface of the agar at right angles to the position formerly occupied by the primary streaks. After further incubation, the test strains will appear as in Pl. 1 B; the growth of the secondary streak is interrupted to a greater or less extent where it crosses the site of any primary streak which had produced an inhibitor against it. In fact, the plate itself presents the results of the test in tabular form.

Technical details

Depth of the agar. The agar should be rather deeper than usual, to prevent the slight inhibition of growth due to local exhaustion of the medium. About 100 ml. of nutrient agar/6 in. Petri dish is a convenient amount, giving a layer of medium 5–6 mm. deep.

Cellophan disks. These should be cut dry, slightly smaller than the surface of the agar, from cellophan about 0·03–0·04 mm. thick. A cellophan which gave satisfactory results with this technique was Cellophane grade P.T. 400 (British Cellophane Ltd., Bath Road, Bridgwater, Somerset). The waterproofed variety of cellophan cannot be used as it is not sufficiently permeable. The disks are washed in distilled water, and several of them may be autoclaved in a Petri dish with sufficient water to prevent them sticking together or becoming warped during sterilization.

Preparation of the plates. A sheet of sterile cellophan is picked up in flamed *but cool* forceps, the excess moisture is allowed to drain off, and the disk is spread out on the agar with forceps and, if necessary, a sterile bent glass rod. If the cellophan is not lying flat, e.g. because it has been gripped with hot forceps, it will gradually curl up during the drying and the plate will be spoiled. The plate should be well dried in any of the usual ways. Laying the lid and inverted bottom separately on parallel glass rods held in a frame in a 37° incubator for 1–2 hr. is satisfactory.

Making the streaks. The primary streaks should not be closer together than about 1·5 cm., so that six can be accommodated with ease on a 6 in. plate. The secondary streaks may, however, be closer together—0·8–0·9 cm. apart is a convenient distance—so that eleven may be made on the same 6 in. plate. The primary streaks should not be made nearer than 0·5–0·7 cm. to the edge of the cellophan, and if the latter shows a 'grain', i.e. irregular fine parallel lines presumably caused during extrusion, they are made parallel to the grain. The plates should be dried for a short time after streaking, otherwise there is a tendency for the bacterial growth to spread.

It is convenient for streaking to place the plate over a paper disk on which guide lines, the appropriate distance apart, have been boldly marked. By adopting such a standard procedure, the exact position of the primary streaks may be recalled at any time during or after the remaining operations.

The following method of making the streaks has been found much more convenient than the usual procedure with a loop. The sealed tip of a Pasteur pipette with a long thin capillary is cut off square, the open end flame-polished by holding in the base of a Bunsen flame, and the whole flame-sterilized. The capillary is then heated about 1 cm. from the end and allowed to bend through nearly a right angle by its own weight. After cooling for a few seconds, the open tip is momentarily immersed in a broth culture of the organism which is to be streaked, and withdrawn before the meniscus has ceased to rise. Using the tip of the capillary like a pen, a metre or more of uniform streak—either continuous or in short lengths—can be traced without having to recharge the capillary (cf. loop technique). When all streaks with that particular organism have been made, the tip of the capillary is cut off just above the highest point to which the broth culture reached, and after flaming and bending, it is ready for the next culture. When the capillary has been used up, another is drawn out from the same pipette. This method (which surely must have been used many times before, though no reference to it could be found) gives a streak which is much more uniform throughout its length than can usually be obtained with a loop.

Limitations of the method

There are certain obvious limitations of the method:

(1) The technique cannot be used for the detection or study of inhibitors which will not diffuse through cellophan, e.g. the products of *B. brevis* (Dubos, 1939), or the protein diplococcin (Oxford, 1944). It may be possible to use instead of cellophan a membrane with larger pores, but still impermeable to bacteria, such as a gradocol membrane or cellophan treated in the manner described by Seymour (1940).

(2) In its present form it cannot be used unless primary and test organisms will both grow on the same medium. The medium used must also, of course, be one on which the antibiotic can be formed.

(3) The method is not suitable for organisms which spread on solid media. Partial success in dealing with such organisms was, however, attained by confining the streaks within ridges of beeswax-plus-resin or beeswax-plus-Venetian turpentine, the wax mixture being applied while hot with a bent Pasteur pipette, in the same way as the bacterial streaks.

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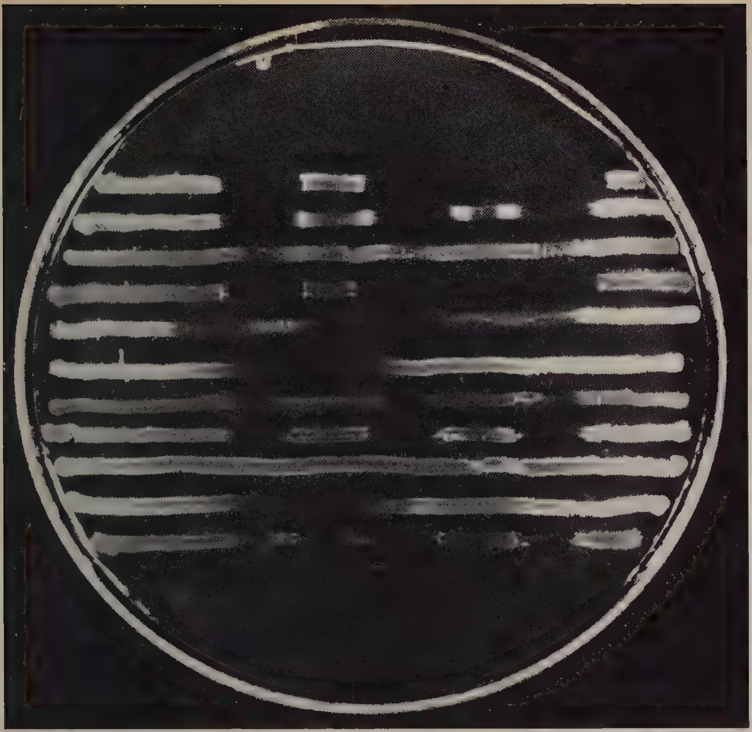
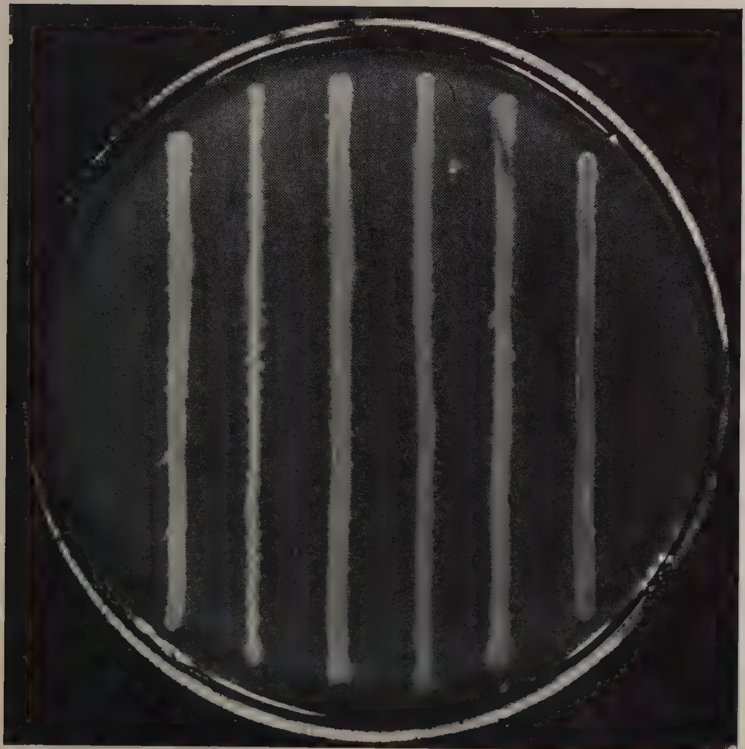


B. Same plate showing fully grown streaks of various test organisms, planted after removal of cellophan sheet.



A. Primary streaks of six different coliform organisms, on cellophan.

N. G. HEATLEY—A SIMPLE PLATE METHOD FOR MULTIPLE TESTS OF THE ANTIBACTERIAL ACTIVITY OF MANY BACTERIA AGAINST OTHER BACTERIAL STRAINS. PLATE 1

Microbiological Aspects of the Submerged-Culture Production of Penicillin on a Pilot-Plant Scale

By E. GRENFELL, B. J. LEGGE and T. WHITE

The Research Laboratories, John Wyeth and Bro. Ltd., London

SUMMARY: The use of mechanical air filtration combined with ultra-violet irradiation of the filtered air has aided markedly in avoiding contamination by undesirable micro-organisms during pilot-plant scale work on penicillin production. By maintaining master cultures of *Penicillium* strains in sterile soil it has been found possible to eliminate strain variation and to maintain the penicillin-producing capacity of the strains. This procedure, followed by transfer of the organism to a rye grain substrate, gives a convenient method of securing large numbers of spores for large-scale work.

It has been found to be desirable to check the purity of the strains, their penicillin-producing capacity and the viability of the spores at various stages of the production sequence; methods have been developed for each of these requirements. Improvements in the cup assay method of estimating penicillin have eliminated certain difficulties encountered in this assay and have enabled the overall error of the method to be reduced to 5%. A colorimetric method of assay has been developed which gives results agreeing with those of the cup assay. With this new method the penicillin content of a sample can be estimated in 5 hr.

Earlier work on penicillin formation showed that this is a particularly intricate microbiological process (Fleming, 1929; Clutterbuck, Lovell & Raistrick, 1932; Abraham, Chain, Fletcher, Gardner, Heatley, Jennings & Florey, 1941). The progress made towards a solution of this problem has remained secret (under wartime regulations) until recently, when groups of workers in the U.S.A. published certain information regarding techniques used in the study of the surface and submerged culture formation of penicillin. These papers, which will be referred to in detail later, do not discuss many aspects of practical importance, and the present communication gives some account of methods used in these laboratories to secure adequate yields of penicillin in submerged culture of the mould on a pilot-plant scale.

The most important practical microbiological problems involved are: (*a*) selecting and maintaining *Penicillium* strains of good penicillin-producing capacity; (*b*) obtaining the bulk of spores required as inoculum for large-scale operations; (*c*) eliminating contamination with other micro-organisms at all stages; (*d*) carrying out the process of penicillin formation under controlled conditions; (*e*) following the course of penicillin formation accurately.

Abraham *et al.* (1941) gave information of practical value on these points for surface culture, but the first detailed statement of basic principles for larger scale operation was that of Foster, Woodruff & McDaniel (1943). These authors showed that, in order to obtain reasonable yields of penicillin, it was necessary to select strains and to avoid the degeneration of penicillin-producing capacity which is so marked in *Penicillium* spp. They stated that such degeneration was best eliminated by carrying master strains in lyophilized soil culture to avoid successive transfers on artificial media, and indicated the importance of using multiple-spore inocula instead of the more normal practice of attempting to establish pure lines by single-spore isolation. Stress was also laid on the value of proceeding from the master-soil cultures to the production stage with the minimum number of intermediate transfers. These points were emphasized by Moyer & Coghill (1946 *a*), who advocated the preservation of cultures by the lyophile method (see Thom & Raper, 1945).

The value of strain selection was discussed in more detail by Raper, Alexander & Coghill (1944), who first described the 'screening test' for assessing the potential value of strains. Agar plugs taken from the surrounds of *P. notatum* colonies grown on modified Czapek's agar were implanted on plates seeded with *Staphylococcus aureus* and the strain value assessed by observing the size of the inhibition zone produced by diffusion of penicillin from each plug. The method had quantitative limitations, but was of great value in eliminating poor strains and was used in the recent isolation of high-potency mutants after irradiation treatment (Raper & Fennell, 1946; Hanson, Myers, Stahly & Birkeland, 1946).

The technique of obtaining large quantities of spores was developed largely by workers at the Northern Regional Research Laboratories, Peoria, and was eventually described by Moyer & Coghill (1946 *a*). They used a 'liquid sporulation medium' which stimulated heavy sporing of *Penicillium* strains, and also described the method in which the organism was inoculated into sterile wholemeal bread or (Moyer & Coghill, 1946 *b*) wheat bran moistened with 2% (v/v) corn-steep liquor solution. The particular application of these methods to the preparation of inocula for deep-culture fermentation has been described by Koffler, Emerson, Perlman & Burris (1945) and by Moyer & Coghill (1946 *b*); these papers also deal briefly with the use of germinated spore suspensions for the inoculation of shake flask, aerated bottle, and aerated tank fermentations. In these laboratories we had been fortunate in having the guidance of G. R. Rettew (private communications) of our associate U.S. company, and acknowledgement should be made of his unpublished work on many of the above problems. In particular, he had used the soil technique for maintaining mycological cultures of commercial importance long before penicillin production was contemplated, and had developed, too, the system of intermediate transfer of master strains to sterile rye grain for obtaining high yields of spores. His advice on sterilization by physical methods was also invaluable.

The problem of avoiding contamination by other micro-organisms during large-scale operations with *Penicillium* has received no attention in the literature, although it was, perhaps, the major problem in such work. Outside the penicillin field, methods of eliminating air-borne and other undesirable micro-organisms have been described by Rahn (1945) and Reyniers (1943). In our work we have used in particular ultra-violet (U.V.) irradiation and mechanical filtration.

The assay of penicillin by the cup method was developed originally by

Abraham *et al.* (1941), and improved by Foster & Woodruff (1943, 1944); it has also been discussed by Schmidt & Moyer (1944), Beadle, Mitchell & Bonner (1945) and by Knudsen & Randall (1945). Despite the care with which these workers described the method they left unmentioned factors which can cause appreciable variation of results, and we found it necessary to investigate the method further and to develop an alternative more rapid method of assay which would give results in agreement with those of the cup assay.

Our methods have been based partly on the above published work, partly on data made available by the penicillin information exchange scheme, and partly on our own investigations. The following details may be of value to workers in this and related fields who, by virtue of the war, have not had access to information which is of some importance. The value of these techniques may be judged from the results described by Gordon, Grenfell, Knowles, Legge, McAllister & White (1947), and by the fact that they were achieved without contamination difficulties in laboratories in which similar large-scale work with a *Bacillus* capable of destroying penicillin was also in progress.

EXPERIMENTAL

Culture media

(1) *Nutrient agar.* Difco peptone, 5 g.; Difco yeast extract, 3 g.; Difco beef extract, 1·5 g.; glucose, 1 g.; 'Davis' agar, 10 g.; distilled water to 1 l.; pH adjusted to 7·2 before sterilizing (6·9–7·0 in final medium). After pouring, plates were incubated at 37° to test sterility.

(2) *Modified Czapek's agar.* $NaNO_3$, 3 g.; KH_2PO_4, 1 g.; $MgSO_4,7H_2O$, 0·5 g.; KCl, 0·5 g.; $FeSO_4,7H_2O$, 0·01 g.; sucrose (brown sugar), 30 g.; 'Davis' agar, 15 g.; corn-steep liquor, 10 ml.; distilled water to 1 l.; pH adjusted to 5·6 before sterilization (5·3–5·9 in final medium). After pouring, plates were incubated at 25° to test sterility.

(3) '*Sporulation*' *agar* (*Moyer slope*). This medium was introduced by Moyer (unpublished work) and is similar to that of Moyer & Coghill (1946 *a*). The composition is as follows: NaCl, 40 g.; KCl, 0·2 g.; KH_2PO_4, 0·06 g.; $MgSO_4,7H_2O$, 0·05 g.; $ZnSO_4,7H_2O$, 0·022 g.; $FeSO_4,7H_2O$, 0·005 g.; brown sugar, 40 g.; 'Davis' agar, 30 g.; molasses, 4 ml.; corn-steep liquor, 4 ml.; distilled water to 1 l.; pH 4·3–5·0 in final medium without adjustment. The agar is omitted if a liquid medium is required.

(4) *Nutrient broth.* Cultures of *Staph. aureus* used as inocula for penicillin assays were grown on this medium, the composition of which was: Difco peptone, 5 g.; Difco yeast extract, 1·5 g.; Difco beef extract, 1·5 g.; glucose, 1 g.; phosphate buffer pH 7·0 (8·4 g. $K_2HPO_4 + 3{\cdot}0$ g. KH_2PO_4/l.), 500 ml.; distilled water to 1 l.; pH 7·0 without adjustment.

The 'sterile room'

Transfers of *Penicillium* were carried out in an adapted part of the laboratory termed, for convenience, the 'sterile room'. Its use was essential for the inoculation technique described later, since contamination by penicillin-destroying organisms would have been inevitable if transfers had been made in the normal laboratory atmosphere. The arrangement of the sterile room is shown in Fig. 1.

An air filter (constructed to design by Airscrew, Ltd., Weybridge, Surrey) set in the inner wall drew air from an outer air-lock through a felt bacterial filter into the inner (inoculating) portion of the room, giving twelve air changes an hour in this area. A strip U.V. lamp ('Hanovia') on the wall opposite the air intake was directed horizontally so that any organisms passing the filter were immediately exposed to U.V. irradiation, which was most intense in the upper third of the inoculating room. The filtered, irradiated air then swept down and out through a grill in the bottom of the inoculating room door into the air-lock. Here it was drawn again to the filter past a second U.V. lamp (directed downwards) above the sterile gowns referred to later. The air in the sterile room was thus continuously circulated, filtered, and irradiated—the inoculating room proper being always under a slight positive pressure. The air-lock contained three previously sterilized gowns and masks hung immediately below the U.V. lamp; these were replaced after using three or four times, or after known contamination. The inner room contained only a stool, enamelled-top table and gas burner.

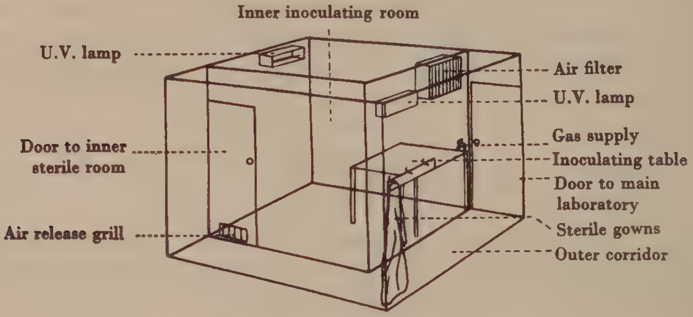


Fig. 1. The 'sterile room'.

The following procedure was adopted in using the sterile room. Cultures and apparatus were placed on a surgical trolley together with thin surgical rubber gloves, 0·1% (w/v) mercuric bichloride solution, absorbent cotton-wool swabs and a spray gun. Two operators entered the air-lock with this trolley, donned the inoculating gowns, masks and gloves, and swabbed the latter with bichloride solution. The trolley, and the surfaces of culture containers and apparatus on the trolley, were then swabbed with bichloride by one operator, while the other sprayed the air-lock and then the inoculating room with calcium hypochlorite solution (0·06% available chlorine). Both operators then entered the inoculating room with the trolley, and reswabbed the gloves, culture containers and apparatus before placing the latter on the swabbed inoculating table. Cotton-wool plugs were then pulled out slightly, flamed, and pushed back into position with a damp swab. Transfers were effected as in normal mycological routine. Pouring, when necessary, was done through a flame unless living organisms were present in the liquid, when it was performed immediately under a flame. The table top was reswabbed and the inoculating room and air-lock resprayed after use.

The efficiency of the arrangement in producing aseptic conditions was tested as follows, three experiments being conducted in each case. The sterile room was first deliberately contaminated by operators in non-sterile clothing, the filter and U.V. lamps having been switched off and the doors opened to the main laboratory atmosphere for an hour.

Two plates each of nutrient agar and modified Czapek's agar were exposed for one hour on the inoculating table, on the window sill of the air-lock and on a central shelf in the main laboratory. The doors of the inoculating room and the air-lock were closed, the filter operated for 24 hr., and a second set of plates then exposed for 1 hr. In one of the three experiments the U.V. lamps were put into operation over a second 24 hr. period and a third set of plates exposed. The room was then sprayed with hypochlorite solution and a fourth set of plates exposed immediately. The Czapek agar plates were incubated at 25° for 3 days, and the nutrient agar plates at 37° for 1 day and then at 25° for 2 days. Mould colonies on the former, and bacterial and yeast colonies on the latter were then counted, and the results for duplicate plates averaged. Table 1 gives the results of these experiments. The contamination of the main laboratory was investigated nine times, giving an average of 336 micro-organisms per plate per hr. (308 bacteria and yeasts plus 28 moulds). More than 90% of the bacterial colonies were *Bacillus brevis*, no doubt as a result of contemporary work with this organism. The degree of contamination of the air-lock and of the inner portion of the sterile room was always decreased to 5–8 and 1–2% respectively of that of the main laboratory at the same time. Since these percentages were obtained after all types of treatment, it was presumed that entry and exit of the operators involved these amounts of contamination, and it was concluded that the filter was efficient in removing micro-organisms from the air circulating in the inoculating room.

Table 1. *Experimental test of the 'sterile room'*

		Treatment											
		None			Filter* (1 day)			Filter (2 days) U.V.† (1 day)			Filter (2 days) U.V. (1 day), spray‡		
Exp. no.	Place	Bacteria	Moulds	Both	Bacteria	Moulds	Both	Bacteria	Moulds	Both	Bacteria	Moulds	Both
1	A	111	44	155	114	8	122	—	—	—	—	—	—
	B	20	17	37	6	4	10	—	—	—	—	—	—
	C	26	27	53	2	1	3	—	—	—	—	—	—
2	A	350	12	362	278	36	314	—	—	—	—	—	—
	B	50	2	52	16	10	26	—	—	—	—	—	—
	C	34	5	39	4	0	4	—	—	—	—	—	—
3	A	494	14	508	891	31	922	192	15	207	314	20	334
	B	117	6	123	41	3	44	6	5	11	14	4	18
	C	96	3	99	22	1	23	2	1	3	5	1	6

A, outer laboratory; B, air-lock of sterile room; C, inner portion of sterile room.

The figures represent the average colony count from duplicate plates exposed for 1 hr. in the places indicated; surface area of plate = *c.* 30 cm.^{2}.

* Air filtration system turned on. † Ultra-violet lamps turned on. ‡ Room sprayed with hypochlorite solution.

In view of the heavy unavoidable contamination of the main laboratories due to pilot-scale antibiotic extraction work, the efficiency of the system is clearly high and its value is shown by the fact that contamination never became a problem throughout the work.

Maintenance and storage of stock cultures

It has already been pointed out that maintenance of the penicillin-producing capacity of *Penicillium* strains is a serious problem. In our work, new strains were subcultured immediately on receipt to provide stock cultures for storage, and to obviate the necessity of opening the original culture too often. If the new strain was received as a soil-spore culture, it was subcultured on a slope of sporulation agar and incubated for 5 days at 25°. Sterile distilled water (5 ml.) was added to this slope culture (or to that of a new strain received in this form) and 1 ml. quantities of the resultant spore suspension transferred to stock soil tubes. These were prepared by the method of Thom & Raper (1945) by autoclaving 3 g. lots of moistened soil at 15 lb. pressure for 20 min. on three successive days. The inoculated soil tubes served as master cultures for the strain and were stored at 4° where they dried out gradually. This process had no adverse effects on *Penicillium* or *Aspergillus* spp., although it was fatal in the case of a *Fusarium* and should not be considered applicable to all fungi. The master soil suspensions were normally used to initiate the first production sequence of the strain (see Chart in the next section), and were then stored for reference. Further sequences were carried out with the second (storage) soil suspension described later. If these gave poorer results than those given by the master soil stock, they were discarded and the latter used again. The technique is safer than continuous transfer on agar, is simpler than the lyophile method of storage, and gave satisfactory maintenance of strain quality.

Stages leading to penicillin production

For the reasons given earlier it was considered necessary to have as few transfers as possible between strain storage and production, or between successive stages of storage. The following chart shows the procedure adopted to obtain a growing and sporulating culture, and to allow of a purity test, a potency test, continuation of storage, and of a large supply of spores for germination and subsequent inoculation of production media.

A loop of soil plus spores was transferred from the master stock culture to a Moyer's agar slope and incubated at 25° for 5 days. Distilled water (5 ml.) was then added and the tube agitated to release the spores. The spore suspension so obtained was used to inoculate one 20 oz. Roux bottle, three plates of modified Czapek's agar, and two rye grain flasks (vide infra). It could also be used to prepare further soil suspensions for storage but we have preferred the alternative method shown.

Roux bottle culture and strain purity

The Roux bottle culture was intended to test the purity of the strain and contained 100 ml. modified Czapek's agar (composition as given earlier except that K_2HPO_4 was used instead of KH_2PO_4, and the pH was 7·2). A loop of spore suspension was spread over the agar surface, larger quantities having proved unsatisfactory as the liquid was absorbed unevenly with consequent effect upon the colonial appearance. After incubation at 25° for 5 days the colonies were examined macroscopically for such morphological dissimilarity as inhomogeneity or sectoring. Changes of this kind usually denoted impurity, or mutation with alteration of the antibiotic properties, and such cultures were stored until subcultures had been taken and tested further.

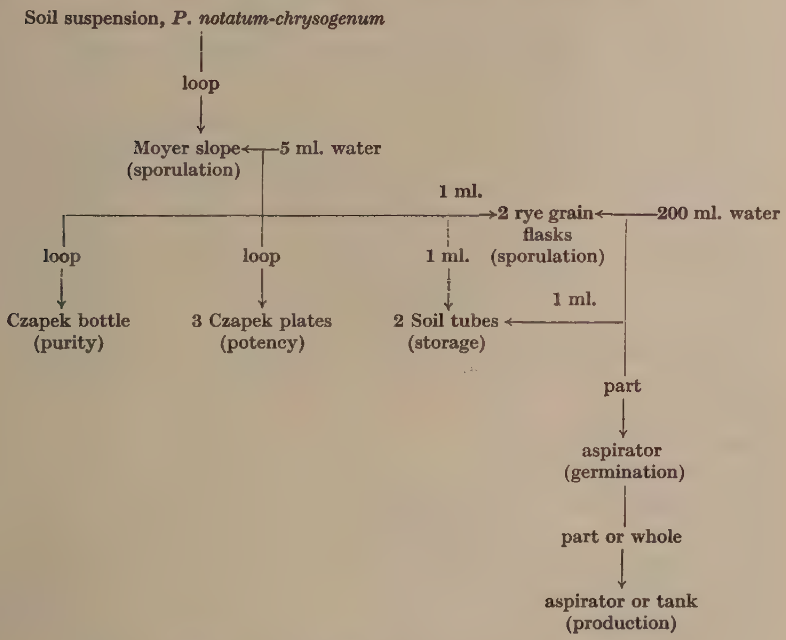

Soil suspension, *P. notatum-chrysogenum*
loop
Moyer slope
(sporulation)
5 ml. water
1 ml.
2 rye grain
flasks
(sporulation)
200 ml. water
loop
loop
1 ml.
1 ml.
Czapek bottle
(purity)
3 Czapek plates
(potency)
2 Soil tubes
(storage)
part
aspirator
(germination)
part or whole
aspirator or tank
(production)

Chart of stages in the submerged-culture production of penicillin

A typical example of the importance of this purity test is seen in the case of a strain of *P. chrysogenum* X1612 which was received as a soil culture from the U.S.A. and gave satisfactory results for some 4 months. A few differently coloured colonies then appeared in a Roux bottle culture and regularly thereafter, the capacity of the strain for producing penicillin in submerged culture diminishing progressively at the same time. Subcultures taken from atypical colonies or colony sectors were tested for capacity to produce penicillin by the plug assay method described later; they were also examined under the microscope. The two most common types of colony were developed through the production stages and grown in submerged culture in aspirator bottles. Subsequent transfers showed the mutant colonies to be stable. The results of these investigations are summarized in Table 2. Plug assay results with the light and dark green colonies were not significantly different and gave no indication of the greater capacity of the latter for producing penicillin in submerged culture, the assay method being qualitative rather than quantitative. On the other hand, the much poorer cream mutant would normally be eliminated by this technique. Microscopic examination revealed no significant difference between the light and dark green colonies, but confirmed the suspected absence of sporulation in the case of the cream colonies. It is clear from this example that routine testing of strains for purity is essential if desirable properties are to be maintained. No conclusions were reached as to the reasons for the deterioration of this particular strain.

Table 2. *Deterioration of a strain of* P. chrysogenum X1612

Origin of variant	Incidence (%)	Colonial appearance	No. of colonies tested	Average diameter of colonies (mm.)	Average diameter of inhibition zone* (mm.)	Penicillin yield in aspirator deep culture (Oxford units/ml.)
Strain before colony variation	100	Light green, wide white border, agar turned yellow	11	23·1	32·6	151
Type A. Light or medium green colonies on Moyer's or Czapek's agar and white parts on nutrient agar	80	As above	27	22·9	31·5	23
Type B. Dark green colonies or sectors on Moyer's or Czapek's agar and brown parts on nutrient agar	20	Dark green, narrow white border, agar very slightly yellow	26	23·2	30·2	34
Type C. Cream, white or pale yellow colonies or sectors on Czapek's agar	<1	Cream, raised	3	13	25·2	Not tested

* Obtained in assays of penicillin-producing capacity by the agar plug technique: the results given are in each case the average of the inhibition zones given by the plugs immediately adjacent to the colonies tested.

Strain potency

Plug assay of the plate cultures (see Chart) was used as an initial check on the potency of the strain before large-scale stages in production were reached, and also as a rough estimate of the value of new strains or of mutants of strains already in use.

Each Petri dish contained 20 ml. modified Czapek's agar (pH 7·2) and was centrally inoculated with a loop of spore suspension from the Moyer's agar slope. (Adjustment of the pH to 7·2 was important since the stability of penicillin decreases below pH 6·0, and it was found that plugs taken from *P. chrysogenum* X1612 cultures on Moyer's agar at pH 4·8 caused no inhibition zones on *Staph. aureus* plates.) After exactly 5 days' incubation at 25°, five 8 mm. plugs were cut with a sterile cork-borer from the agar immediately adjacent to the mould colony. The plugs were transferred without inversion, by means of a spear-head needle, to an assay plate seeded with *Staph. aureus*, and placed at equidistant points about 25 mm. from the centre. These assay plates were prepared and seeded as described later for the normal penicillin assay. After implantation the assay plates were incubated at 37° for 16 hr. and the diameters of the clear circular inhibition zones around the plugs measured. As the size of the zones depends on the amount of penicillin diffusing from the plugs, a rough estimate could be made of the potency of the mould strain concerned.

The power of a surface colony to produce penicillin on an agar plate did not, however, always bear a strict relationship to the capacity of the strain for producing penicillin in submerged-liquid culture. For this reason, therefore, the plug assay was looked upon only as a convenient test for eliminating poor strains, and for routine checking of each strain prior to its use for large-scale work. Raper *et al.* (1944) used plugs cut from the mould colony itself, and from the agar in a radial series toward the edge of the plate. Plugs taken from the actual colonies of highly potent strains give zones over 40 mm. in diameter and, in Petri dishes, these are distorted and are difficult to measure accurately. Furthermore, plugs equidistant from the same colony give variable results. We therefore considered it better to take five plugs of the latter type giving measurable zones of inhibition, and averaged the results to cover variation. The differences between individual values are presumably due to variable diffusion, or to variable penicillin production by different arcs of the colony.

Rye grain culture, sporulation, and spore viability

The use of rye grain as a substrate for fungal growth is due to our colleague G. R. Rettew (private communication) although the procedure has been modified to suit our needs. Liquid sporulating medium (25 ml.) was added to dry rye grain (25 g.) in a plugged 500 ml. conical flask and autoclaved at 15 lb. pressure for 20 min. Each such flask was inoculated with 1 ml. spore suspension from the Moyer's agar slope and well shaken. After 7 days' incubation at 25° most of the rye grain surface was green with spores and the culture could be used immediately for making a spore suspension, or stored at 4° for periods up to a fortnight. Spore suspensions were prepared by adding distilled water and shaking to release the spores. In early experiments a sample was taken for counting and the bulk used for inoculation without further treatment. Spore aggregation, however, rendered inaccurate both the counting and the subsequent measurement of the inoculum, and a crude filtration process was therefore included. A circular piece of muslin was hollowed into and wired over the mouth of a 500 ml. conical flask, the top of which was then covered with a wad of non-absorbent cotton-wool and greaseproof paper. After the assembly had been sterilized, the spore suspension was poured through the muslin under cover of the cotton-wool cap. Total counts were carried out with ten samples of the filtered material using a haemocytometer chamber of volume 0·005 mm.^{3}. The final suspension normally contained $3\text{–}7 \times 10^7$ spores/ml., different times of incubation of the rye grain culture and a varying efficiency of filtration significantly affecting the count. One ml. lots of the suspension were transferred to soil tubes which were stored at 4° for the purpose already described.

In order to establish the optimum conditions for penicillin production on a large scale it was necessary to be able to estimate the number of growing fungal colonies per unit volume of medium. As an essential stage of each production sequence, germination of a measured quantity of the spore suspension was carried out in a 10 l. aspirator bottle containing production medium (Gordon *et al.* 1947), and the problem was therefore to determine what proportion of the spores germinated under these conditions. This aspect appears to have received no attention from other workers.

Table 3. *Viability of* P. chrysogenum *spores*

Strain	Culture used as source of spore suspension	Samples counted	Spores germinated / Spores inoculated	Germination (%)	Possible range*
X1612	5-day Moyer slope stored 14 days at 4°	50	1465/10,440	14	3–100
X1612	7-day rye grain stored 2 days at 4°	25	615/3748	16	11–21
X1612	7-day rye grain	25	2658/6793	39	23–79
X1612	8-day rye grain	10	1173/4603	25	20–33
Q176	7-day rye grain stored 3 days at 4°	35	1775/3995	45	19–72
Q176	8-day rye grain	25	6810/17,616	39	18–55
Q176	7-day rye grain	25	1303/6374	21	15–35

* Range of percentage germination when calculated from the lowest and highest total spore counts rather than from the mean.

A portion of the muslin-filtered suspenion was diluted with four volumes of water, mixed and divided into two aliquots. One of these was used for determining the total count as above. According to the results obtained a further dilution of the second portion was made and an aliquot added to a known amount of modified Czapek's agar at 47°. After mixing well, the agar was distributed (10 ml. quantities) in Petri dishes which were then incubated at 25°. Colony counts were made on the second day and repeated on the third day, but not thereafter since sporing had started and secondary colonies were a possibility. The results were expressed as the percentage of the spores inoculated which germinated.

The conditions of the test were necessarily different from those in an aerated liquid production medium, but significant differences in germination were not expected since essential conditions were supplied in both cases. Results obtained with *P. chrysogenum* X1612 and Q176 are given in Table 3. The 'possible range' (see Table 3) was particularly wide in the first investigation in which no filtration of the spore suspension was included and spore aggregation resulted in very variable counts. The viability of the spores of these strains of *P. chrysogenum* was much lower than anticipated, being in all cases less than 50%, and this had to be taken into account when calculating the size of inocula for production purposes. In view of these results, we feel that determination of spore count and spore viability of inocula is desirable for proper control of penicillin fermentations.

Aspirator bottle culture and germination of spore inocula

Germination of the spores used as inocula for small- and large-scale penicillin fermentations was accomplished in 10 l. Pyrex glass aspirators. The general assembly for this purpose was similar to that used for work on tyrothricin in these laboratories (Appleby, Knowles, McAllister, Pearson & White, 1947). Normally, 7 l. of the medium (the same as that used in the subsequent production run) was made up in the aspirator assembly and autoclaved at 15 lb. for 30 min. Spore suspension of known spore content was added through the inoculation inlet and the assembly incubated in a thermostatically-controlled room at 27·5 or 29·5°. Owing to the aeration, the temperature of medium was 2·5° lower than that of the room. Aeration was normally at a rate of 1 l. air/l. medium/min., and no agitation other than that due to aeration was used. Initial frothing of the medium during incubation was decreased by adding, before sterilization, 1–3 ml. antifoam (2% stearyl alcohol in lard oil). After 24–30 hr. excessive frothing generally occurred and 1–5 ml. lots of antifoam were again added through the inoculation inlet. The exit of air under pressure at this point minimized the possibility of contamination during this operation. Aspirator cultures for the germination of spore inocula for tank-scale fermentations were normally grown for 48 hr. and then used as described by Gordon *et al.* (1947).

Germinated cultures intended for the inoculation of further aspirator runs were grown for 2–4 days and then removed to the sterile room. The original level of the culture medium was restored by adding distilled water and, with knowledge of the initial spore number and of the percentage spore viability, the number of colonies per unit volume of the culture was estimated. The contents of the aspirator were well shaken and the required volumes removed through the sampling tube into sterile graduated flasks for inoculating the production aspirator runs. The apparatus and mode of operation for these was exactly the same as that used for inoculum germination. The results of such experiments are described by Gordon *et al.* (1947).

The purity of cultures was checked at each stage of the production sequence by plating on modified Czapek agar and on nutrient agar.

The assay of penicillin

The cup method assay

The procedure used was similar in essentials to that described by other workers (vide supra), but varied in certain details.

The cups used were of aluminium (10 mm. × 6 ± 0·1 mm. internal diam.: 8 mm. external diam.) with one end bevelled from the inner to the outer surface. Moisture condensation and consequent spreading of the test organism was prevented by using

Petri dishes (10 cm. diam.) with porcelain Coor lids; these contained 21 ml. nutrient agar. A further quantity of the same agar was melted, cooled to 47°, and inoculated (1 ml. to 100 ml. medium) with a 16 hr. culture of *Staph. aureus* (N.C.T.C. 6571*a*) in nutrient broth. Seeded agar (4 ml.) was distributed evenly over the surface of each of the plates, which were then ready for use in the present method or in the plug assay method described earlier. Five cups were placed symmetrically on each plate; to ensure a good seal they were dropped on with forceps from a height of *c.* 3 mm. immediately after the agar had set. Standard penicillin solution (0·2 ml., 1 Oxford unit/ml.) was measured into cups 1 and 3, and the same volume of the solution under test (adjusted to approximately the same penicillin content) to cups 2, 4 and 5. The plates were incubated for 16 hr. at 37°, and the diameters of the inhibition zones read to the nearest 0·5 mm. The strength of the unknown was read from a standard curve which had been obtained from experiments in which the five cups contained solutions of standard penicillin of concentrations 0·4, 0·6, 0·8, 1·0 and 1·3 Oxford units/ml. The standard curve was adjusted up or down the 'zone diameter' axis according to the plate variation indicated by the inhibition zones given on each plate by the two cups containing standard penicillin.

Table 4. *Effect of various factors on the assay of penicillin by the 'cup assay' method*

Factor varied	Normally	Experimentally	Average change in zone diameter (% of normal)	No. of zones measured
Age of *Staph. aureus* inoculum (hr.)	16	22·5	+4·4	156
Temperature at which agar inoculated (°)	47	56	+10·5	262
pH of agar	6·9	6·35	+7·3	265
Delay before incubating plates after filling cups (min.)	30	60	+4·5	105
Volume in cups (ml.)	0·2	0·1	−11·0	79

Solid standard penicillin (Provisional British Standard) was stored at 1° and stock solutions (50 Oxford units/ml.) in ether-saturated water prepared every fortnight. These were further diluted as required with phosphate buffer pH 6·0 (K_2HPO_4, 3·25 g.; KH_2PO_4, 10 g.; water to 1325 ml.). Test solutions were passed through a bacterial filter to remove mycelium and diluted with the same buffer to *c.* 1 Oxford unit/ml. All solutions were kept cold until used.

Penicillin concentrations were calculated only from results obtained with test solutions containing from 0·9 to 1·1 Oxford units/ml. In order to avoid variation and possible contamination of the test organism (*Staph. aureus*) a routine check of the condition and purity of the test culture was made before each assay. Stock cultures on nutrient agar slopes were stored at 4°.

In early assays high average zone sizes and diffuse zone edges were sometimes encountered. As this tended to make the assays inaccurate, some investigation of the factors responsible was carried out. The use of Difco yeast extract in the assay medium produced sharp edges, while other yeast extracts tested gave diffuse edges. The effect of other factors on zone diameter is shown in Table 4; conditions which discouraged growth of the test organism caused an increase of zone size. The effect of varying several factors simultaneously was additive with the added disadvantage that there was a tendency towards indefinite diffuse zone edges.

The effect of varying the volume of solution placed in the cups is shown in Fig. 2. Other workers have specified only that the cups should be 'filled'. Increasing the volume added from 0·2 to 0·3 ml. gave an increase of 7·0% in the inhibition zone diameter. Errors of this order could therefore occur if the term filled' is laxly interpreted and we consider it essential to use a standard volume. To obtain consistent results it is essential to adhere rigidly to the standard conditions of assay described, particularly as far as temperatures, pH values, and times are concerned. The overall error under these conditions is of the order of 5% only.

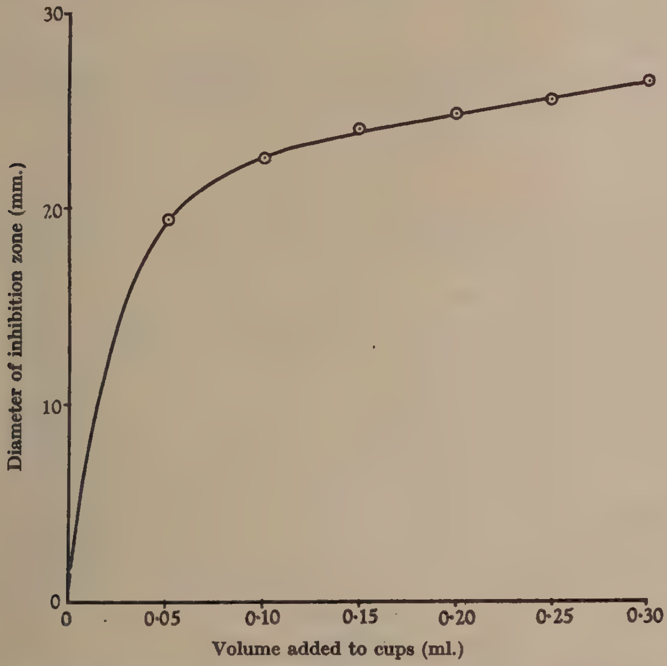


Fig. 2. Variation of diameter of inhibition zone with volume added to cups. Penicillin solution contained 1 Oxford unit/ml. (Provisional British Standard.)

The colorimetric broth assay method

This method was devised in order to provide an alternative and more rapid method of assay which would give results in agreement with those of the cup assay. It was adapted from that developed for the assay of tyrothricin by Appleby, Knowles, Pearson & White (1947). The method is based on the graded inhibition by increasing quantities of penicillin of the growth of *Staph. aureus* in nutrient broth, acid production being used as a measure of growth.

Standard penicillin was diluted to 0·2 Oxford unit/ml. and the unknown to approximately the same concentration. The volumes shown in Table 5 were then measured into two series of 3 × ½ in. tubes (one series for the standard and the other for the unknown). Water was added to each tube to bring the volume to 1 ml. Papain heart digest broth (Appleby, Knowles, Pearson & White, 1947) containing 1% (w/v) glucose and 3% bromcresol-purple indicator solution (British Drug Houses Ltd.) was mixed with one-twentieth of its volume of a 16 hr. broth culture of *Staph. aureus*, and 1 ml. mixture added to each assay tube. The latter were then inverted once to mix, and incubated in a water bath at 37° for 4 hr. The result was obtained by comparing tubes of the two series in which inhibition of growth was partial, i.e. those in which the indicator showed an intermediate colour change. Tubes from the unknown and

Table 5. *Colorimetric broth assay of an unknown penicillin solution*

	Tube										
	1	2	3	4	5	6	7	8	9	10	11
Standard *or* unknown penicillin solution (ml.)*	0·0	0·1	0·2	0·3	0·4	0·5	0·6	0·7	0·8	0·9	1·0
Penicillin present in 'standard' series (Oxford units)	0·0	0·02	0·04	0·06	0·08	0·10	0·12	0·14	0·16	0·18	0·20
Growth:†											
'Standard' series	+	+	+	+	±	±	±	±	±	±	−
'Unknown' series	+	+	+	±	±	±	±	±	±	−	−
Penicillin in matching standard (Oxford units)	.	.	.	.	.	0·12	.	0·16	.	0·20	.
Penicillin in undiluted unknown (Oxford units/ml.)	.	.	.	.	.	240	.	229	.	222	.

* Standard, 0·2 Oxford unit/ml.; unknown, 1/1000 dilution of solution under test.

† + signifies indicator turns yellow, and − that it remains purple; ± represents intermediate changes.

Table 6. *Comparison of two methods for the assay of penicillin*

	Penicillin found (Oxford units/ml.)	
Sample	Colorimetric broth assay	Cup assay
1	40	40
2	142	141
	143	—
3	225	218

standard series were matched visually, and the results calculated from the average given by three or four matching tubes (Table 5). The overall error of the method was judged to be *c.* 15%. Table 6 shows that the results were in close agreement with those obtained by the cup assay method; turbidimetric methods, on the other hand, usually tend to give higher values. Aseptic precautions were taken throughout, but this is possibly not essential in view of the short incubation period.

Using this method, the penicillin content of a production sample can be estimated within 5 hr., and the course of penicillin production can be followed more closely than would be otherwise possible.

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Methods of Penicillin Production in Submerged Culture on a Pilot-Plant Scale

By J. J. GORDON, E. GRENFELL, E. KNOWLES, B. J. LEGGE, R. C. A. McALLISTER and T. WHITE

The Research Laboratories, John Wyeth and Bro. Ltd., London

SUMMARY: This paper gives details of a 50 gal. fermentation vessel designed for investigating the formation of antibiotics (or other metabolic products) by micro-organisms grown in submerged culture. This vessel has been used for investigating the submerged culture production of penicillin by *Penicillium chrysogenum* X1612 and Q176, and certain results relating to the size of the inoculum and the yields obtainable from these strains in synthetic and other media have been obtained. Culture fluids containing from 400 to 500 Oxford units penicillin/ml. have been obtained with cultures of Q176 in a corn-steep liquor medium.

A method of extracting penicillin from the broth has been worked out, based on solvent transfer, the method being applicable on virtually any scale of operation and involving only relatively simple equipment. It has the advantage of reducing the time of contact of penicillin with acid to such a degree that extraction at room temperature is possible, although extraction at still lower temperatures improves the yield. Using this method of extraction we have obtained calcium penicillin with a potency of 940 Oxford units/mg., the overall recovery from the broth being of the order of 35–50%.

The large-scale production of penicillin by deep culture methods became possible largely as a result of the development of suitable strains of the mould by methods such as those described by Raper, Alexander & Coghill (1944) and by Raper & Fennell (1946). The development of corn-steep liquor lactose media by Moyer & Coghill (1946*a*), and of synthetic media by the Pennsylvania State University workers (unpublished work) made feasible the proper utilization of these strains. The technique and biochemistry of penicillin formation in submerged culture has been investigated by Koffler, Emerson, Perlman & Burris (1945), Knight & Frazier (1945), Koffler, Knight, Emerson & Burris (1945), Raper & Fennell (1946), Foster, Woodruff, Perlman, McDaniell, Wilker & Hendlin (1946), Moyer & Coghill (1946*b*), Foster, Woodruff & McDaniell (1946) and Raper *et al.* (1944). These workers have been concerned partly with aspects already discussed in the preceding paper (Grenfell, Legge & White, 1947); with establishing, by shake-flask, aerated bottle, and aerated tank experiments, the optimum conditions for penicillin production; and with investigation of the metabolic changes during the course of the experiments. Stefaniak, Gailey, Brown & Johnson (1946) describe the construction and operation of pilot-plant scale fermentation vessels for experimental investigations, and this is the only published information on this aspect. It is felt therefore, that some account of the techniques used in our laboratories, and of the results obtained, may be of value. These techniques have, of course, been based to some extent on the above-mentioned work, details of which were made available before publication by the official penicillin information exchange scheme.

Since the results now presented are intended mainly to illustrate the value of the methods and equipment described, it is not proposed to discuss in detail the metabolic investigations covered by the above communications. They provide a basis for understanding the effects of changes in medium composition, and of variation of operating conditions, upon the yield of penicillin. The use of high potency strains under optimum conditions and the addition to the medium of substances specifically stimulating penicillin formation, has resulted in penicillin yields of *c.* 500 Oxford units/ml. culture fluid in 70–80 hr. Yields approaching this level have been obtained in our work on a 200 l. scale although in a slightly longer period.

Developments in the extraction of penicillin have received little attention in the literature. Two main methods are used: (*a*) adsorption of penicillin on carbon, elution with organic solvents, concentration, and purification by solvent transfer; (*b*) solvent transfer alone, usually in the sequence culture fluid, organic solvent, aqueous buffer, organic solvent, aqueous alkali; the last solution being used for freeze-drying. We have developed for solvent transfer a convenient technique which can be used on any desired scale.

EXPERIMENTAL

Methods and equipment

Analytical methods

The course of each fementation was followed by periodic determinations of pH (electrometrically); sugar utilization (method of Schaffer & Hartman, 1920); ammonia content (micro-Kjedahl); and penicillin content (Grenfell *et al.* 1947). Other features could, of course, have been followed. Over a period of time, however, it was found that changes in the above constituents constituted the data of greatest significance and that from consideration of pH, sugar, and ammonia values it was normally possible to predict whether or not a fermentation was proceeding satisfactorily, and the time at which the culture fluid should be processed to obtain the best yield of penicillin. These two aspects are naturally of importance for production.

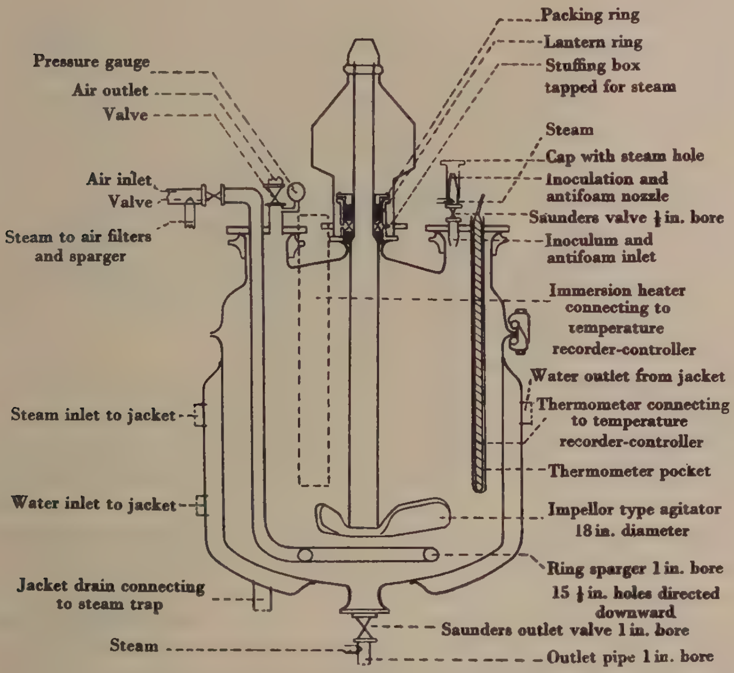
Culture media

Synthetic media No. 22A. This medium was developed for use in penicillin production by the Pennsylvania State University group of workers (unpublished). The composition is: lactose B.P., 15 g.; glucose B.P., 5 g.; acetic acid (glacial), 4 g.; NH_4NO_3, 5 g.; KNO_3, 3·5 g.; KH_2PO_4, 2 g.; $MgSO_4,7H_2O$, 0·5 g.; $FeSO_4,7H_2O$, 0·2 g.; $ZnSO_4,7H_2O$, 0·04 g.; $CuSO_4,5H_2O$, 0·005 g.; phenylacetamide, 0·25 g.; water to 1 l.

Corn-steep liquor medium. The composition of this medium is: corn-steep liquor (Stahley no. 14), 30 ml.; lactose B.P., 40 g.; $CaCO_3$, 10 g.; phenylacetamide, 0·25 g.; water to 1 l. Antifoam (300 ml./200 l. medium) is added before sterilization.

Apparatus for small-scale experiments

Small-scale experimental submerged-culture fermentations were carried out in 10 l. aspirators each containing 7 l. medium. The aspirator assembly and mode of operation are described by Grenfell *et al.* (1947) and, for similar work on tyrothricin, by Appleby, Knowles, McAllister, Pearson & White (1947)


Packing ring
Lantern ring
Stuffing box tapped for steam
Pressure gauge
Air outlet
Valve
Steam
Cap with steam hole
Inoculation and antifoam nozzle
Air inlet
Valve
Saunders valve ½ in. bore
Steam to air filters and sparger
Inoculum and antifoam inlet
Immersion heater connecting to temperature recorder-controller
Water outlet from jacket
Steam inlet to jacket
Thermometer connecting to temperature recorder-controller
Thermometer pocket
Water inlet to jacket
Impellor type agitator 18 in. diameter
Ring sparger 1 in. bore 15 ¼ in. holes directed downward
Jacket drain connecting to steam trap
Saunders outlet valve 1 in. bore
Steam
Outlet pipe 1 in. bore

Text-fig. 1. Diagrammatic vertical section of pilot-plant apparatus for penicillin production.

The fermentation vessel

The fermentation vessel used for pilot-scale runs was basically a normal Pfaudler S-type jacketed glass-lined reaction kettle of capacity 50 gal. This was modified slightly and fitted with accessory equipment designed specially for deep culture work on penicillin and tyrothricin. The main details are shown in Text-fig. 1 and Pl. 1. The vessel cover carried five ports, the largest of which was fitted with a cover carrying a light and sight glass and acted as a chargehole. The central 4 in. port was fitted with a stirrer and the three remaining 4 in. ports carried respectively (*a*) thermometer pocket and nozzle for injecting inoculum and antifoam; (*b*) air sparger and air outlet; (*c*) immersion heater. The chargehole is not shown in Text-fig. 1 and the position of the heater is merely indicated. The bottom of the vessel had a 3 in. opening fitted with a 1 in. Saunders glass-lined valve plus sampling pipe. Either steam or water could be circulated through the outer jacket, the supply pipes (positions shown in Text-fig. 1) being controlled by valves. The steam exit led to a steam-trap system for draining off condensate.

Agitation. Agitation was by a glass-coated three-bladed stirrer of the 'Impellor' type with extreme blade diameter 18 in. (3 in. greater than the sparger diameter) and revolving at 150 r.p.m. The stirrer blades were sited *c.* 3 in. above the sparger and were angled at 45° from the vertical, thus causing a downthrow of the medium into the air stream. The stuffing-box through which the stirrer was inserted into the fermenter was specially designed to prevent the possible entry of contaminating bacteria. In addition to the normal rings of packing material, the stuffing-box contained a perforated steam lantern ring (Text-fig. 1). The side of the stuffing-box was drilled and tapped at the level of this ring and a steam line inserted into the tapping; a similar tapping at the diametrically opposite point carried a fine steam exit-jet. Steam was passed continuously through the lantern ring throughout operation of the fermenter, the fine exit causing an increase in pressure and thus maintaining a sterilizing zone around the stirrer shaft. As an added precaution, the packing rings were lubricated with grease containing 1% creosote.

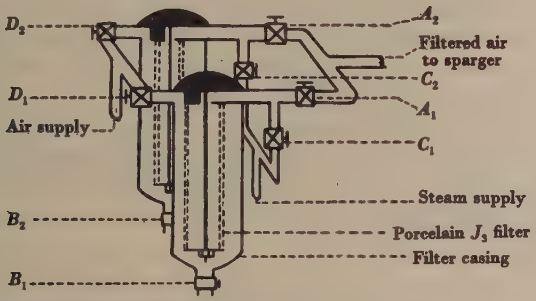
Temperature control. The fermenter was fitted with an immersion heater intended mainly for bacteriological fermentations. Unlike Stefaniak *et al.* (1946), however, we found that heating was required in the early stages of penicillin fermentations and the heater proved advantageous. The heater was linked externally to a 'Temperature Recorder-Controller' (Cambridge Instrument Co.) which was connected also to a metal thermometer inserted in the thermometer pocket of the fermenter. The controller governed the temperature (normally 25°) throughout the fermentation, and also provided a continuous record of the temperature inside the vessel. The immersion heater, being 3 in. in diameter, further served as a baffle aiding adequate air dispersion and preventing clumping of the mycelium.

During rapid mycelial growth the temperature tended to rise above 25°. This was counteracted by running cooling water through the jacket of the fermenter, the automatic action of the heater-controller system preventing the temperature dropping below 25°. Control was secured to 0·5° by these means.

Aeration. Air was provided by a compressor-after cooler-air receiver system of capacity 250 l. free air/min. at 40 lb. pressure. The air passed through a 1 in. pipeline, pressure fluctuations being smoothed out and the pressure reduced to 30 lb. by a reducing valve. After this was a stop-cock and branch pipe for use when adding inoculum or antifoam to the vessel. The main air line then entered a vertical 'Rotameter' calibrated up to 250 l. free air/min. (allowing for subsequent filter resistance, medium resistance, and the pressure drop to that of the atmosphere).

After leaving the 'Rotameter' the air was freed from micro-organisms by passage through two 'Aerox' air filters fitted with grade 'J3' porcelain filters of pore size 1·5μ. The details of the arrangement are shown in Text-fig. 2;

provision was made at this point for admission of steam to the circuit. The air line led finally into the 1 in. mild steel air sparger of the fermenter, terminating inside the vessel as a horizontal circular air sparger of 15 in. diam., centrally placed about 2 in. above the bottom of the vessel and 3 in. below the stirrer vanes. This circular portion had 15 uniformly spaced downwardly directed holes ($\frac{1}{8}$ in. diam.) which distributed the air evenly into the medium. Air left the tank by a 2 in. exhaust line fitted with a pressure gauge and a steam valve fitment for controlling the pressure inside the fermenter. This was normally 5–10 lb. to minimize the possibility of contaminating organisms entering the fermenter during the runs.



Text-fig. 2. Arrangement of air filter system. A_1, A_2, 1 in. Saunders glass-lined valves; B_1, B_2, filter case drain cocks; C_1, C_2, 1 in. gate valves on steam lines; D_1, D_2, 1 in. gate valves on air lines.

Sterilization of the fermenter, air-supply and medium

Materials sufficient to make up 200 l. medium were added to 180 l. tap water in the fermenter. All openings were then bolted down and the valves closed. The following valves were then opened in sequence (Text-figs. 1, 2): (i) the inoculation nozzle valve; (ii) the air outlet valve of the fermenter; (iii) the steam exit valve leading from the jacket to the steam-trap; (iv) the air line valves A_1, A_2; (v) the drain cocks B_1, B_2 of the filters; (vi) the steam valves C_1, C_2. The last action caused steam at 30 lbs. pressure to pass simultaneously back through both porcelain filters, and forward through the air line and sparger into the medium. The porcelain filters were thus sterilized while the fermenter was being sterilized and thereafter were capable of passing sterile air for tested periods of up to 300 hr.

Steam was next turned on at the jacket to speed up the heating of the medium. When the medium temperature had risen to 100° and steam was passing freely from the inoculation nozzle (*c.* 25 min.) the air outlet valve of the fermenter was closed until the fermenter pressure reached 15 lb. and the valve then adjusted to maintain that pressure. Sterilization was carried out for 30 min. and the inoculation valve then closed, steam being turned on above this valve as described later. Steam was then turned off at the jacket and the steam valves C_1 and C_2. At the same time the valves D_1, D_2 were opened and the cocks B_1, B_2 closed, thus allowing air to pass through the filters into the fermenter. Adjustment of the air exit valve of the fermenter was then required to maintain the 15 lb. pressure. Normally, to avoid undue evaporation losses, aeration at this stage was limited to 40 l./min., this being sufficient to maintain at least 5 lb. pressure in the fermenter during the cooling process. Cold water was then turned on at the jacket and the medium temperature brought down to 25° in 90 min.; the tank was then ready for inoculation. The temperature control system was then brought into operation and, after inoculation, the air intake adjusted to the desired rate (1 l. air/l. medium/min.). During the sterilizing process, the volume of medium usually rose to the desired 200 l. as a result of steam condensation during the heating stage. The use of 'Aerox' porcelain filters was more convenient than the U.S. practice of using cotton- or glass-wool filters and gave efficient air sterilization without other accessory equipment and without undue mechanical bulk. The two parallel filters were capable, under our conditions, of passing up to 250 l. air/min. with the backing pressure of 30 lb. They were so inserted that either filter could be detached during the course of a fermentation, the porous element changed, steam sterilized, and then brought back into operation without interrupting the fermentation.

Inoculation

The inoculation nozzle of the fermenter served both for the addition of the inoculum and for the subsequent addition of antifoam agent (2% stearyl alcohol in lard oil). Its construction can be seen from Text-fig. 1. Particular attention was paid to the design of this item since contamination was most likely to be introduced at this point. The nozzle consisted of a tube of $\frac{1}{2}$ in. bore entering through a port cover and carrying (as close as possible to the outside of the cover) a $\frac{1}{2}$ in. glass-lined Saunders valve. The portion of the nozzle above the valve had a $\frac{1}{4}$ in. steam line inserted as close as possible to the valve seating: it was threaded to take a cap immediately above this point, and then tapered to a 2 in. long portion of $\frac{3}{8}$ in. bore at the inlet end. This section was covered by a removable threaded winged cap with a lateral $\frac{1}{16}$ in. hole to act as a steam exit.

During sterilization of the fermenter, the inoculation valve was open and the cap of the nozzle screwed on so that steam at sterilizing pressure passed from the tank through the inoculation system and emerged via the lateral hole in the cap. This effectively sterilized the inlet route of the inoculum. Shortly before completion of the sterilization of the fermenter, the inoculation valve was closed and steam turned on at the nozzle steam line to maintain sterile conditions above the valve until the time of inoculation. When the fermenter had been cooled to the operating temperature, this steam was turned off, the winged cap unscrewed and removed, and an aspirator containing germinated spores (Grenfell *et al.* 1947) immediately connected to the nozzle by sterile rubber tubing which was wired in position, both to the nozzle and to the tube normally serving as the aspirator air outlet. The branch air line (vide supra) was then connected by rubber tubing to the air filter tube of the aspirator and the latter clamped in an inverted position above the fermenter. The fermenter pressure was then lowered to 2 lb. by operating the air exit valve, the inoculation valve opened, and air at 30 lb. pressure passed from the branch air line into the aspirator, thus forcing the inoculum into the fermenter. On completion of this process the air passing into the aspirator was turned off, the inoculation nozzle valve closed, and the aspirator disconnected from the nozzle. The nozzle cap was then refitted and steam turned on at the nozzle steam line to resterilize the system from the valve seating upwards. The fermenter pressure was finally readjusted to 5–10 lb.

A previously sterilized aspirator assembly containing antifoam was then attached to the nozzle and additions of antifoam made when required by the same technique, except that the assembly was left attached to the fermenter throughout the run.

Sampling

The 1 in. bottom exit valve of the fermenter terminated in an outlet (3 in. long × 1 in. diam.) with a steam line inserted immediately below the valve seating (Text-fig. 1). The end of the outlet was threaded to take a cap. The outlet and lower face of the valve seating were steamed continuously during fermentations. The steam was turned off immediately before sampling, the outlet valve opened slightly and closed immediately a sufficient sample had been obtained. Enough sample was first run to waste to cool the outlet to a temperature which would not affect the penicillin content of the actual sample used. After sampling the valve was closed at once and steaming recommenced.

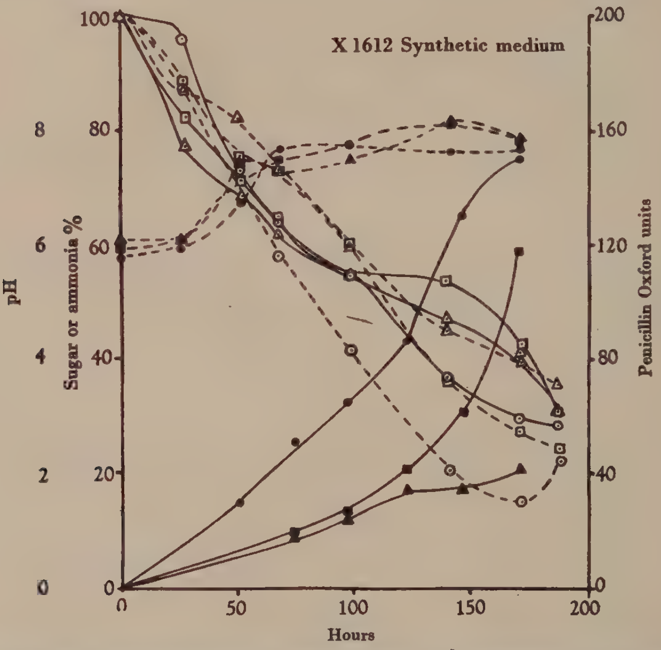
Occasionally the valve became blocked with mycelium. In this case the valve was shut, the outlet pipe capped loosely and steam passed through the outlet pipe via the loose cap for 30 min. Steaming was then stopped, the cap tightened, and the outlet valve opened momentarily while the steam was simultaneously turned full on, thus forcing steam into the fermenter. With quick careful operation this procedure freed the blocked valve without detrimental effect on the fermentation; any other procedure would have involved contamination.

The use of steam sterilization at this point, at the stirrer insert, and at the inoculation nozzle proved highly successful and made it possible to operate without contamination throughout the experiments.

The formation of penicillin

A large number of experiments were carried out both on the aspirator and the tank scale, in which different conditions of fermentation, different media, and different strains were tested. The tank work was done with two U.S. strains (X1612 and Q176) of *P. chrysogenum.* A comparison was made of the behaviour of these two strains in media based on corn-steep liquor, and in synthetic medium. It is proposed to discuss here only certain aspects of the results exemplifying points of importance which have not received previous comment or emphasis.

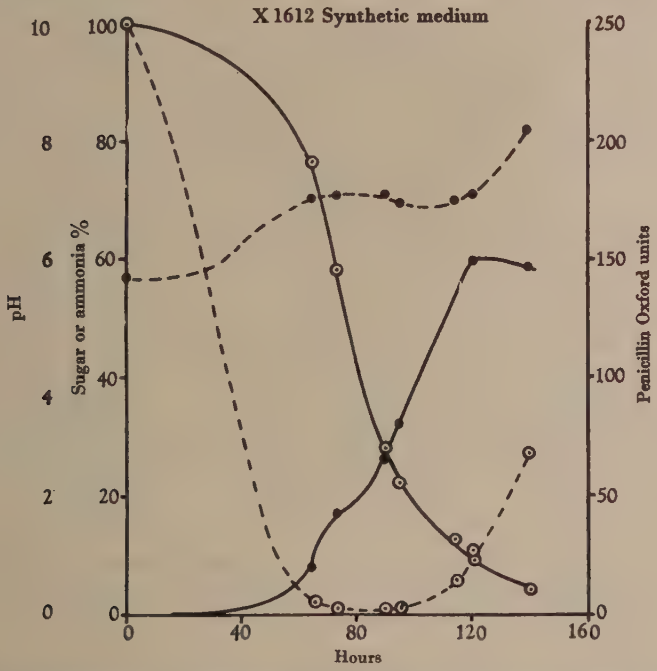
Text-fig. 3 illustrates the effect of inoculum size on the yield of penicillin (strain X1612, synthetic medium). It has been our practice to inoculate at the final stage with a culture which had already been grown for 48 hr. One of us (T. W.) had found in the U.S.A. considerable disagreement as to the volume of inoculum that should be added, figures given ranging from 0·5–10% of the volume of medium. The variability of spore viability from strain to strain (Grenfell *et al.* 1947) may account for this disagreement. Our experiments were designed to determine the best inoculum size in terms of viable spores rather than as volume of inoculum only. The three experiments of Text-fig. 3 were carried out simultaneously and show that the heavier the inoculum, the greater was the initial utilization of sugar and ammonia, and the less was the final yield of penicillin. In the end, however, the smallest inoculum actually gave the greatest utilization of sugar and ammonia, and also the most rapid production of penicillin and the highest yield (155 Oxford units/ml.). The results suggest, in fact, that too large an inoculum uses up so much nutrient for mycelial growth that penicillin formation is badly affected either by prolongation of the initial growth phase or by the creation of unfavourable conditions. This and later findings suggested that for strain X1612 under the



Text-fig. 3. Effect of inoculum size on the metabolism of *P. chrysogenum* X1612 in synthetic medium on an aspirator scale. Inoculum (germinated spores): 5×10^5, ⊙ ●; 5×10^6, ⊡ ■; 10^7, △ ▲. Actual volumes of inoculum were 25, 250 and 500 ml. respectively. Sugar utilization (%), —⊙— etc.; ammonia utilization (%), --⊙-- etc.; pH, --●-- etc.; penicillin (O.u./ml.), —●— etc.

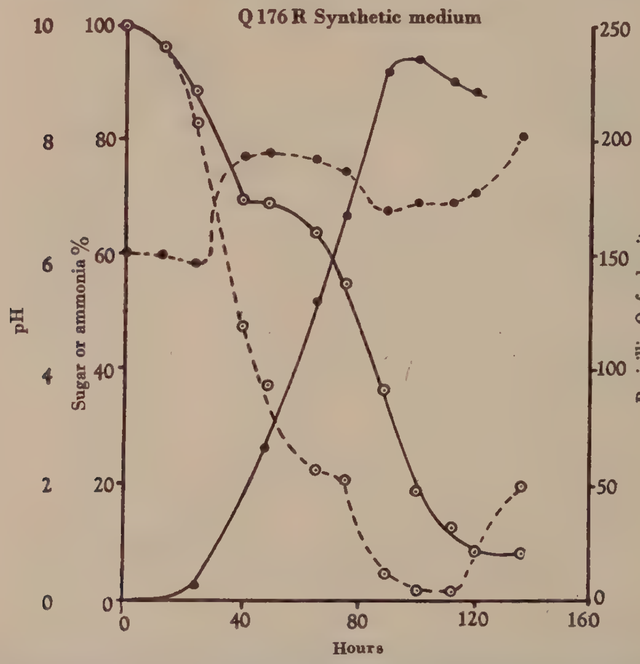
conditions of these experiments the inoculum should be 10^5 germinated spores/l. medium. For different strains, media, and operating conditions the figure would probably be different and the aspect is one which we feel merits closer attention.

Text-fig. 4 shows the results of a comparable experiment with the same strain and medium in the pilot-plant. A much faster fermentation results under the more efficient conditions of aeration, agitation, etc., that were possible on this scale. This particular run is chosen because it illustrates clearly the correlation of the time of the peak penicillin yield with the marked final rise of pH and ammonia content of the medium. In practice, this serves as an excellent indication of the time when the cultures in this medium should be processed for optimum yields of penicillin and, since both determinations are more quickly carried out than any penicillin assay yet devised, the point is of some pratical importance. Strain Q176R behaved similarly in this respect in this medium (Text-fig. 5), the peak yield being 245 Oxford units/ml. It is clear also that the advantage of this strain over X1612 lies not in a more complete utilization of nutrients, but in the fact that there is a much shorter lag phase before penicillin formation begins, while the rate of formation is much



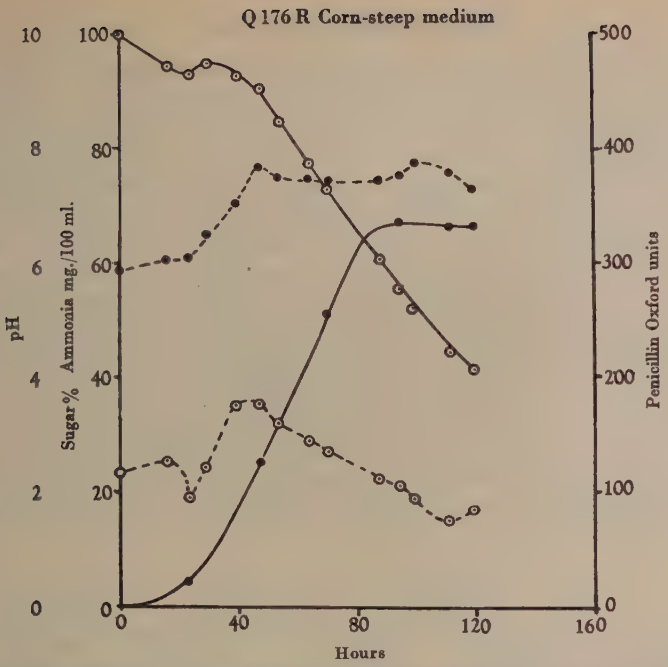
Text-fig. 4. Metabolism of *P. chrysogenum* X1612 in synthetic medium on a pilot-plant scale. Sugar utilization (%), —⊙—; ammonia utilization (%), -- ⊙ --; pH. -- ● --; penicillin (O.u./ml.), —●—.

faster and the final level higher. There is a clear distinction too in the initial pH changes, strain Q176 showing a more definite and faster initial pH rise than X1612. Our experience with this medium has been that the greater and more rapid this early rise, the higher is the yield of penicillin. The final point of interest in this experiment is the form of the sugar utilization curve. The initial fast rate is due to rapid metabolism of the glucose present, this being followed by a decided lag before lactose is used; this point will receive comment later.

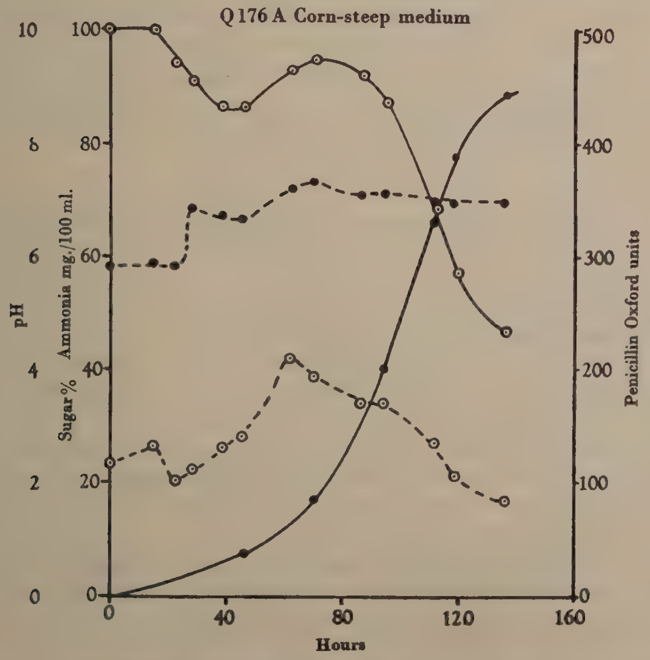


Text-fig. 5. Metabolism of *P. chrysogenum* Q176R in synthetic medium on a pilot-plant scale. Symbols as in Text-fig. 4.

The results of experiments in which corn-steep liquor medium was used in the pilot-plant are shown in Text-fig. 6 (strain Q176R) and Text-fig. 7 (strain Q176A). These two strains were separate isolates from the same original stock. The general similarity between the fermentations is evident, particularly as regards the changes in pH and ammonia content. These curves are very different from the corresponding curves for the synthetic medium. The pH reached an early peak value and then remained approximately constant at a value eminently suitable for penicillin formation, while the ammonia values showed an initial drop (not recorded by other workers) followed by a marked rise and then an equally marked fall with a tendency to rise shortly after the peak penicillin value had been attained. This medium was of particular value not only because it gave high yields (400–500 Oxford units/ml.) but also because



Text-fig. 6. Metabolism of *P. chrysogenum* Q176R in corn-steep liquor medium. Symbols as in Text-fig. 4, except that ammonia values are given as mg./100 ml. medium.



Text-fig. 7. Metabolism of *P. chrysogenum* Q176A in corn-steep liquor medium. Symbols as in Text-fig. 6.

the yields did not fall off very rapidly after the peak—a point of decided value in processing and probably due to the pH remaining constant at a value at which penicillin can persist.

The main difference between the two strains Q176R and Q176A was in their metabolism of sugar and their yield of penicillin. Initially both attack the corn-steep liquor carbohydrate, and this is followed by a lag before lactose metabolism begins. The lag is much more pronounced with Q176A, and penicillin formation is correspondingly delayed. This strain A incidentally shows the same lactose lag in synthetic media. On the other hand the peak penicillin yield is significantly higher with Q176A than with Q176R, which ran consistently to 330 Oxford units/ml. despite slight medium modifications. Foster, Woodruff, Perlman, McDaniell, Wilker & Hendlin (1946), who have provided the only other comparative data on X1612 and Q176, have also commented on the lag in lactose metabolism and advocated adaptation of the strains to lactose to obtain maximum effectiveness. We found also that crude lactose prolonged the lag still more, but increase of quality above the B.P. standard did not affect the lag, nor did the addition of inorganic salts to the medium.

The extraction of penicillin

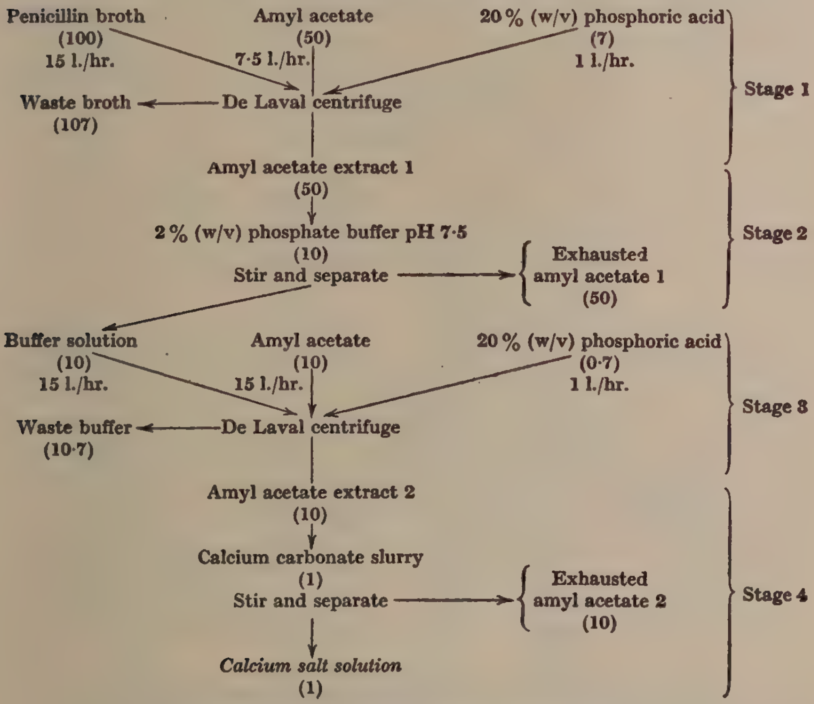
The extraction of penicillin by laboratory scale methods becomes rather difficult with large volumes of medium. Batchwise extraction is undesirable from several points of view and we have therefore developed a method whereby the transfer of penicillin from aqueous solution to organic solvent is carried out by a continuous extraction process in which the time of contact of penicillin with acid is decreased to such a minimum that operation without refrigeration is possible even with volumes of 200 l. The general procedure is shown in the Chart on p. 199. As an example of the use of this method of extraction the following details based on the treatment of a batch of Q176 broth of penicillin content *c.* 400 Oxford units/ml. may be considered:

Stages 1 *and* 2. Penicillin broth, amyl acetate, and 20% (w/v) phosphoric acid were each placed in Pyrex aspirators fitted with stoppers carrying an open glass tube extending to the bottom of the aspirators to provide a constant head of liquid. The lower aspirator outlet had a stopper carrying a delivery tube fitted with an adjustable screw clip; this, in conjunction with the constant head of liquid, enabled constant set rates of flow to be obtained. The broth flow was set at 15 l./hr., the acetate flow at half that value and the acid flow at that figure (predetermined by titration) which would bring the broth to pH 2·0–2·1 during the extraction.

A De Laval no. 1200 laboratory-type centrifuge was set up with the bowl assembled as shown in Text-fig. 8, i.e. with a lower clarifying disk but an upper disk arrangement as for separation—a combination of the two methods of assembly normally used with this machine. With this arrangement, two (or more) liquids run in simultaneously are first thoroughly mixed and emulsified, and then separated as the emulsion passes up through the bowl. The normal clarifier assembly would carry out the first function but not the second, while the normal separator assembly would perform the second function but would not mix and emulsify efficiently.

The bowl was primed with broth, the centrifuge started, and broth, solvent, and acid fed simultaneously into the machine without prior mixing. The acid flow was then finally adjusted until the pH of the discharging broth was 2·0–2·1,

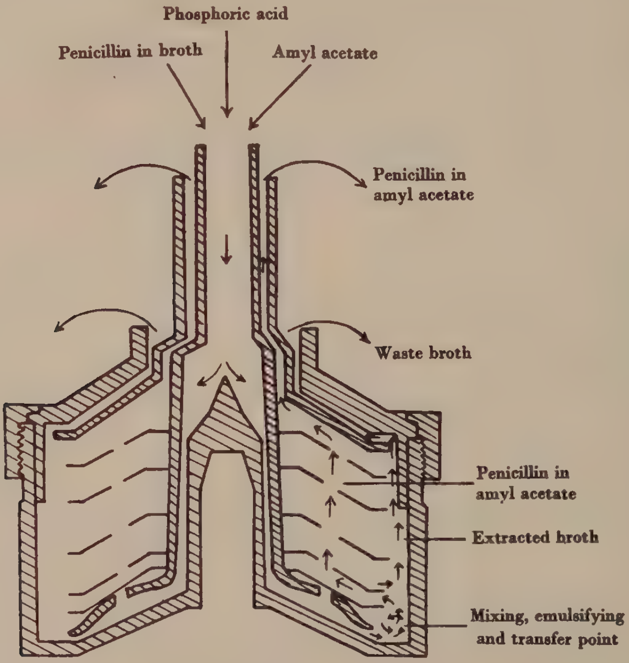
Chart showing extraction procedure.


Penicillin broth
(100)
15 l./hr.
Amyl acetate
(50)
7·5 l./hr.
20 % (w/v) phosphoric acid
(7)
1 l./hr.
Stage 1
Waste broth
(107)
De Laval centrifuge
Amyl acetate extract 1
(50)
2 % (w/v) phosphate buffer pH 7·5
(10)
Stir and separate
Exhausted
amyl acetate 1
(50)
Stage 2
Buffer solution
(10)
15 l./hr.
Amyl acetate
(10)
15 l./hr.
20 % (w/v) phosphoric acid
(0·7)
1 l./hr.
Stage 3
Waste buffer
(10·7)
De Laval centrifuge
Amyl acetate extract 2
(10)
Calcium carbonate slurry
(1)
Stir and separate
Exhausted
amyl acetate 2
(10)
Stage 4
Calcium salt solution
(1)

(The numbers in brackets indicate volumes in l.)

the machine being then run continuously until the first stage of extraction was complete. The process resulted in efficient transfer of penicillin from the broth to the amyl acetate, the waste broth now containing only *c.* 1 Oxford unit/ml. penicillin. The amyl acetate was discharged into a stainless steel vessel containing 2% (w/v) phosphate buffer pH 7·5 (one-tenth of the volume of the original broth). This solution was stirred continuously while the flow of amyl acetate continued, thus securing immediate transfer of the penicillin from the organic solvent phase to a neutral aqueous phase where its stability was high. The pH of the buffer was tested periodically during the addition of the amyl acetate solution; it should not be allowed to fall below 7·1, and more buffer should be added if necessary. The solution of penicillin in buffer was finally run off through a cock at the bottom of the apparatus.

The method had the advantage that the time of contact of penicillin with acid conditions (both in aqueous and organic solvent phases) was a maximum of 1 min.; the time of contact with the acid aqueous phase was only *c.* 10 sec. In consequence it was possible to conduct the process at room temperature without appreciable acid decomposition of the penicillin, the recovery to the buffer stage being of the order of 65–70% even at 20–25°. A typical broth containing 440 Oxford units/ml. (13 Oxford units/mg.) gave a buffer solution of concentration 3000 Oxford units/ml. (150 Oxford units/mg.)—a twelvefold initial purification and tenfold concentration.


Phosphoric acid
Penicillin in broth
Amyl acetate
Penicillin in amyl acetate
Waste broth
Penicillin in amyl acetate
Extracted broth
Mixing, emulsifying and transfer point

Text-fig. 8. Special method of assembly of the bowl of the De Laval centrifuge.

Stages 3 *and* 4. The technique used for these two stages was similar to that used in stages 1 and 2 except that the buffer solution from stage 2 replaced the original broth, and the amyl acetate flow was the same as the buffer flow. The amyl acetate was discharged continuously into a stirred slurry of calcium carbonate (20 g. carbonate plus 500 ml. water/100 l. initial broth). After all the amyl acetate had been run in, the mixture was stirred for a further 10 min. at 1500 r.p.m. and the solution of calcium salt separated off; the pH of the latter was normally 6·1. The amyl acetate solution was then treated as above with two further lots of calcium carbonate slurry (20 g. carbonate in 300 ml. water/100 l. initial broth; 20 g. carbonate in 200 ml. water/100 l. initial broth). The second calcium salt solution was usually pH 7·0, and the third pH 7·2. The distribution of penicillin between the three fractions, and the potency of each is shown in Table 1. The greatest part of the recovered penicillin was in the first solution. Recoveries of the order of 60–70% were again obtained at this stage, the overall recovery for the whole process being 35–50%. Pooling of all three calcium salts gave a final solution of concentration *c.* 15,000 Oxford units/ml., and potency 800 Oxford units/mg. This final solution was suitable for experimental freeze-drying and gave a penicillin of higher potency than has been used for clinical work until recently.

Table 1. *Details of calcium salts obtained at the final stage of penicillin extraction*

Calcium salt fraction	Penicillin content		% of total penicillin
	O.u./ml. solution	O.u./mg. dry wt.	
1	20,000	940	68
2	12,000	600	23
3	6,000	780	9

The isolation of penicillin of this degree of purity on a laboratory scale and without refrigeration proves the value of the simple extraction method used above. Other experiments showed that the use of refrigeration at the two acid transfer stages markedly improved the yield and this, of course, is in accord with large-scale practice. On the small scale, however, the above procedure provides a simple and convenient technique for investigating the effects of variations in the extraction procedure. The continuous flow method could have been applied also to the transfer of penicillin from organic solvent to aqueous neutral buffer. This was not usually done, however, since the transfer can be carried out at temperatures up to 25° without loss of penicillin beyond that due to its distribution coefficient between the two solvents.

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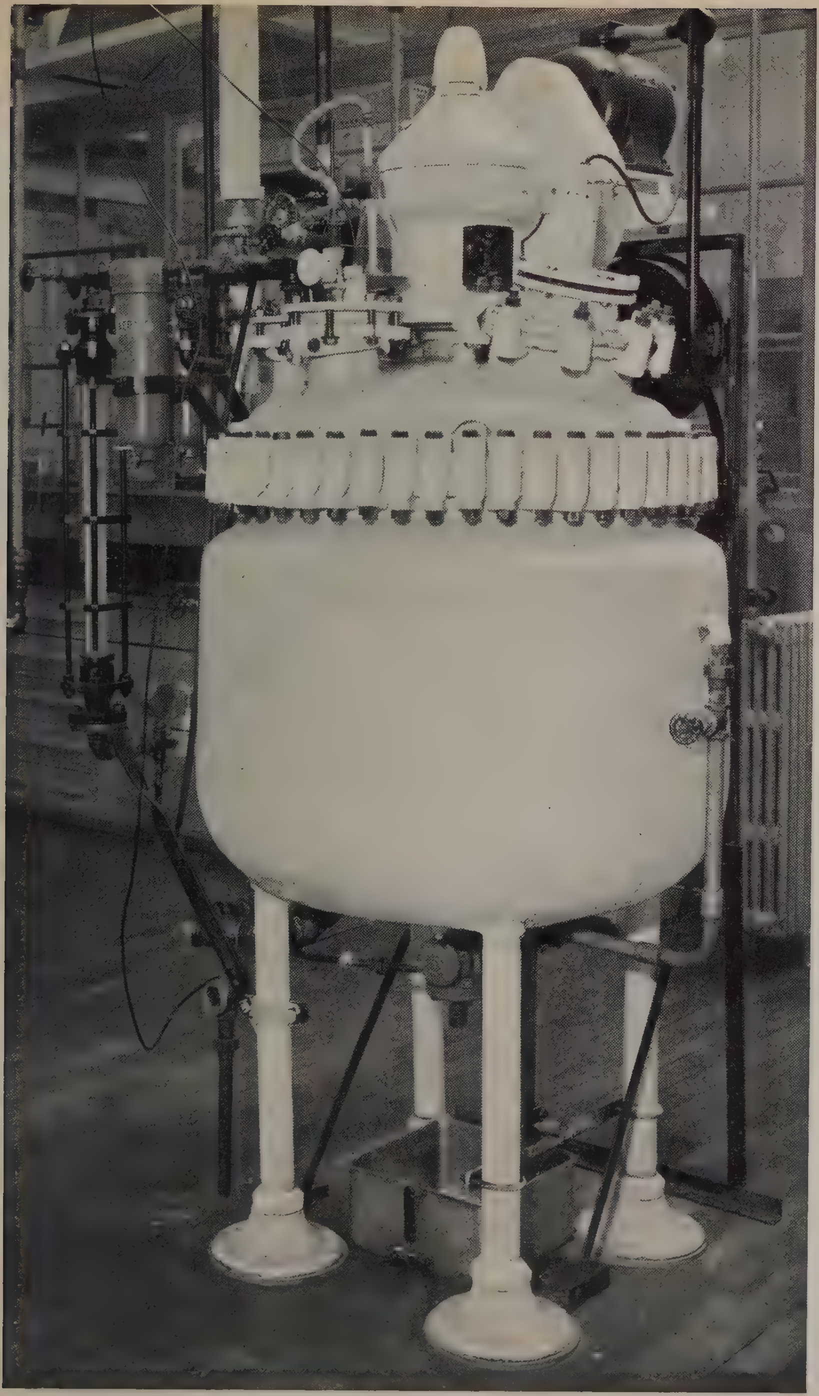

50 gall. pilot-plant penicillin fermenter.

J. J. Gordon, E. Grenfell, E. Knowles, B. J. Legge, R. C. A. McAllister and T. White—Methods of penicillin production in submerged culture on a pilot-plant scale. Plate 1

Clostridium aurantibutyricum (n.sp.): A Pink Butyric Acid *Clostridium*

By ESTHER HELLINGER

Daniel Sieff Research Institute, Rehovoth, Palestine

SUMMARY: A pink, butyric acid-producing *Clostridium* isolated by Ch. Weizmann from South African hibiscus stems is described. It ferments sucrose, glucose, lactose, maltose, galactose, xylose, starch, but not cellulose, inulin, mannitol, glycerol and sorbitol.

The main products of fermentation of maize and glucose are butyric and acetic acids; acetone, butanol, ethanol and *iso*propanol are produced in slight or moderate amounts, lactic acid, formic acid and acetylmethyl-carbinol only in slight or negligible amounts. In its partial diastatic action on maize mashes and in the composition of its resultant metabolic products the new organism resembles members of the so-called butyric group of clostridia; but it differs markedly in liquefying gelatin.

In certain morphological and physiological features the organism resembles two orange-coloured clostridia, *Cl. felsineum* and *Cl. roseum* (both belonging to the so-called butylic group), but is readily distinguishable from them. The two butylic organisms have a complete diastatic action on maize and butanol is the chief product of fermentation. The new organism is also distinguishable by its inability to ferment inulin and to disintegrate potato slopes.

On the basis of this evidence the organism is considered to be a new species, for which the name *Cl. aurantibutyricum* is suggested.

A pink organism, isolated by Ch. Weizmann in May 1939 from a sample of South African hibiscus stems, produced a distinct red coloration on maize mash, gave red colonies on nutrient glucose agar and produced butyric and acetic acids, and small quantities of neutral solvents from sugar, for the complete fermentation of which chalk was required. It produced spores which withstood 2 min. heating at 100°.

A number of these cultures, designated W 45, was investigated after they had been sealed for six to seven months. Some were still viable when subcultured in yeast glucose medium at 37°, but none survived heating for 1–2 min. at 100°, nor for several minutes at 80°. Cultures in maize mash showed active gassing, a definite pink coloration and a good although incomplete diastatic action. The so-called head of solid particles of mash, brought to the surface by the active evolution of gas, sank when the gassing had subsided. The cultures consisted of actively motile rods and clostridia bearing subterminal spores. On yeast glucose agar slopes under anaerobic conditions more or less regular, orange-pink granular colonies were produced (Pl. 1, A). Preliminary experiments showed that the organism dissimilated carbohydrates, producing good yields of butyric and acetic acids and slight amounts of neutral volatile products, such as acetone, butanol, ethanol and *iso*propanol. These data, and especially the prevailing acidic nature of the fermentation products, tend to align the pink organism with the so-called butyric clostridia rather than with the butylic bacteria.

Chromogenic clostridia are not unknown. Two red chromogens have been described, viz. *Clostridium felsineum* Carbone & Tomolato (Carbone & Tomolato, 1917) a true retting organism from retting flax, and *Cl. roseum* McCoy & McClung (McCoy & McClung, 1935), found occasionally with *Cl. acetobutylicum.* Because of their special ability to produce high yields of acetone and butanol from starch mashes these two chromogens are grouped with the butylic organisms. No record of a pink butyric organism could be found in the literature except one by McClung (1942) who reported the isolation of some pigmented butyric types from soil and mud. This, naturally, stimulated a close study of the morphology, cultural aspects and physiological properties of the new pink butyric *Clostridium*, with special reference to its taxonomy and nomenclature.

DESCRIPTION OF THE ORGANISM

Morphology

Vegetative cells. Motile, medium and long rods; rounded ends; singly and in short or fairly long chains. Spore-bearing cells motile, mostly spindle-shaped (Pl. 1, B). Good sporulation at 30° on 5% maize, but scanty at 37°, becoming negligible after several subculturings. Tendency for better sporulation on 1% than 5% maize at 37°.

Endospores. Subterminal and oval.

Table 1. *Measurements of vegetative cells, etc., on yeast glucose agar*

	Limits of size		Average size	
	Length (μ.)	Width (μ.)	Length (μ.)	Length (μ.)
Rods:				
(1) 30°	2·8– 8·0	0·55–0·6	4·7	0·58
(2) 37°	4·0–17·0	0·5 –0·55	9·4	0·51
Clostridia	5·5–10·5	0·9 –1·1	6·9	1·06
Spores	1·9– 2·4	0·85–1·1	2·18	0·97

Flagella. Peritrichous.

Staining reactions. Young cultures Gram-positive. Lugol's iodine: young vegetative cells stain yellow, granulose in clostridia stains blue-violet.

Fermentation of carbohydrates and related substances

The ability of the organism to utilize various substances as a source of carbon was determined by measuring the amount of gas and volatile acid produced from 25 ml. of medium containing 20% yeast water, 0·5 g. of the substance under test, and 0·5 g. calcium carbonate in long narrow tubes (28 × 1·7 cm.) connected with water-filled gas burettes. The results are shown in Table 2.

Table 2. *Fermentation of various carbohydrates, alcohols and glucosides (7 days at 37° and 30°)*

	Gas production (ml.)		Volatile acid (ml. 0·1 N)	
	37°	30°	37°	30°
Glucose, lactose, sucrose, maltose, galactose, levulose, arabinose, xylose, mannose, starch	195–270	185–300	27·5–42·5	29–41·5
Raffinose, rhamnose, dextrin	105–120	95–160	28–35·5	22–27·5
Salicin, pectin (0·3 g.)	47–73	42–97	18–18·5	14–17
Glycerol, inulin, glycogen, mannitol, sorbitol, erythritol, cellulose	0–5	0	3–6	1–6·5
Basal medium + inoculum	—	—	3·5	6·0

Fermentation of glucose. Analyses of the fermentation products of glucose in the presence and absence of calcium carbonate are given in Table 3. The inoculant was an active 24 hr. maize-tube culture. In the first series, 2% glucose was added to 600 ml. of 0·5% Difco yeast extract; in the second series 800 ml. of the same medium were taken, whereas in the third and fourth series 3% glucose was added to 600 ml. of yeast medium with the addition of 20 g. of separately sterilized calcium carbonate.

Table 3. *Products of fermentation of yeast glucose medium after 6–7 days' incubation at 37°*

	Without $CaCO_3$		With $CaCO_3$	
	I	II	III	IV
Glucose fermented (%)	60·38	45·86	100	100
Titratable acidity (ml. 0·1 N acid/10 ml.)	6·4	6·89	—	—
	Products calculated in mmol./100 mmol. of glucose fermented			
Butyric acid	49·4	60·6	43·5	42·1
Acetic acid	51·4	59·4	47·8	63·2
Lactic acid	0·0	9·4	23·9	7·1
Formic acid	0·3	2·7	0·6	1·4
Acetone	3·2	1·8	1·0	1·5
*iso*Propanol	5·4	6·2	3·0	1·6
Butanol	11·9	8·5	7·1	7·4
Ethanol	34·2	45·9	15·8	14·6
Acetylmethylcarbinol	5·8	Traces	0·3	0·6

Residual sugar was determined according to Lehmann-Maquenne-Schoorl (van der Haar, 1920).

Neutral solvents were distilled off from 200 ml. of neutralized fermented medium. 100 ml. of distillate were collected. The acetone content was estimated according to Goodwin's modification (Goodwin, 1920) of Messinger's method; *iso*propanol, by the method of Langlykke, Peterson & McCoy (1935) and ethanol and butanol by Johnson's (1932) method.

Volatile acids were determined from 50 ml. of the fermented medium acidified with phosphoric acid and distilled until about 12 ml. remained. CO_2-free distilled water (50 ml.) was then added to the distilling flask and distillation continued. This was repeated until all volatile acids were distilled over. The volatile acids were then determined according to Duclaux. The distillation constants were calculated from our own data obtained with pure acids.

Lactic acid was estimated directly on the fermented medium by the Fincke method (Official Methods, 1935).

Acetylmethylcarbinol was estimated on 200 ml. of the fermented medium according to Kluyver, Donker & Visser 't Hooft's (1923) modification of Lemoigne's method.

Fermentation of maize mash

5% maize mashes were fermented by the organism at 37° and 30°. The inoculant used was 1 ml. of a young active culture. The analyses of the products of fermentation are recorded in Tables 4 and 5.

Table 4. *Products of fermentation of* 5% *maize mashes at* 37°

Series I and II: 600 ml. fermented mashes after 6 days. Series III: 200 ml. fermented mash after 7 days.

	I	II	III
% starch fermented	82·79	64·22	48·69
Titratable acidity (ml. 0·1 N acid/10 ml.)	6·89	7·40	6·25
	% on fermented starch		
Butyric acid	9·14	15·11	22·88
Acetic acid	9·49	11·33	11·99
Lactic acid	6·49	2·5	—
Formic acid	0·03	0·17	—
Acetone	5·44	1·30	0·19
*iso*Propanol	1·81	0·98	1·41
Butanol	9·42	3·12	0·0
Ethanol	5·02	7·17	6·23
Acetylmethylcarbinol	0·29	0·23	—
Total neutral solvents	21·98	12·80	7·83

Table 5. *Products of fermentation of* 200 *ml.* 5% *maize mash after* 7 *days at* 30°

	I	II
% starch fermented	61·85	53·37
Titratable acidity (ml. 0·1 N acid/10 ml.)	8·8	8·3
	% on fermented starch	
Butyric acid	25·46	18·30
Acetic acid	13·35	23·71
Acetone	0·71	3·09
Butanol	3·00	6·04
Ethanol	11·88	6·15
*iso*Propanol	—	0·78
Total neutral solvents	15·59	16·06

The results of the analyses show that the main products of the fermentation of maize are butyric and acetic acid. It is evident, therefore, that the organism cannot be classified as a true butylic organism; more evidence on this matter is given below, in the discussion of its systematic relationships.

Butyric acid and acetic acids are also the chief products of dissimilation of glucose. Fair amounts of ethanol are produced, small amounts of butanol, and slight amounts of acetone, *iso*propanol and acetylmethylcarbinol. The organism produces more ethanol in the absence of calcium carbonate. Complete fermentation of the glucose takes place when the acids produced are neutralized.

In interpreting the above data for the identification of the new chromogen, it will be observed that the organism has many characteristics in common with both the two closely related specific groups: the butyric group represented by *Cl. butyricum* Prazmowski, and the so-called butylic group represented by *Cl. acetobutylicum* Weizmann, including the two known chromogens *Cl. felsineum* and *Cl. roseum*. The new organism resembles more closely the former group in morphology and in producing butyric acid rather than butanol; but it is a gelatin liquefier and none of the true butyrics is known to liquefy gelatin. Since the liquefaction of gelatin has been used so far as a diagnostic feature of the butylics, one cannot lightly consider the new organism as a pigmented variety of *Cl. butyricum*, nor is there sufficient justification for regarding it as a pigmented variety of *Cl. acetobutylicum*.

COMPARISON WITH RELATED SPECIES

To facilitate the identification of the pink organism, it is compared with the closely related butylic chromogens *Cl. felsineum* and *Cl. roseum*.

The salient characteristics common to all three chromogens are briefly. (1) pinkish to reddish pigmentation on nutrient agar; (2) orange to reddish discoloration of maize and potato mashes; (3) liquefaction of gelatin; (4) the fermentation of pectin to a certain extent; (5) production of acetone, butanol, butyric acid and acetylmethylcarbinol.

The features that distinguish the new chromogen from *Cl. felsineum* and *Cl. roseum* are: (1) its partial diastatic action in maize mash, as against the complete diastatic activity of the butylics; (2) its appreciably lower yields of neutral fermentation products from carbohydrates; (3) its inability to ferment inulin; (4) its failure to disintegrate potato tissue, whereas *Cl. felsineum* completely digests potato tissue to a yellow slime, producing abundant gas and a butyl odour, and *Cl. roseum* behaves similarly to *Cl. felsineum* but forms a clear yellow liquid and bluish sediment; (5) its inability to reduce nitrates; (6) its optimum temperature of growth at 30° as compared with 37° for *Cl. felsineum* and *Cl. roseum*; (7) certain appreciable differences in pigmentation.

A closer comparison of the individual fermentation products provides further evidence of a difference between the three organisms. In Tables 6 and 7, the yields of the major fermentation products obtained by the new organism W 45 in a number of experiments are compared with those recorded for *Cl. roseum* and *Cl. felsineum* (Kluyver strain). The fact that W 45 is a poor solvent producer from maize mash is clearly shown.

The production of *iso*propanol and acetylmethylcarbinol is noteworthy. W 45 produces *iso*propanol from carbohydrates, whereas, according to McCoy and co-workers, *Cl. roseum* and *Cl. felsineum* do not. Van der Lek (1930), also working with the Kluyver strain of *Cl. felsineum*, found *iso*propanol in a few instances only. McCoy and co-workers do not record the production of acetylmethylcarbinol by *Cl. felsineum* and *Cl. roseum*; Van der Lek, however, observed its production from glucose by *Cl. felsineum*. The new chromogen forms it too. Besides the results recorded in Tables 3 and 4, three confirmatory flask experiments were made with 100 ml. of a 2% glucose nutrient medium. Two of the flasks containing calcium carbonate gave 3·4 and 6·5 mg. and the third flask without calcium carbonate gave 3·2 mg. of acetylmethylcarbinol.

Table 6. *Fermentation of 5% maize by* W 45, Cl. roseum* *and* Cl. felsineum* *at* 37°

	W 45					*Cl. roseum*	*Cl. felsineum*
	1	2	3	4	5		
Total solvents (g./l.)	1·38	2·48	1·58	1·44	2·19	11·7	12·2
	Composition of neutral solvents as % of total solvents						
Acetone	2·17	7·07	11·75	3·48	0·00	27·6	30·5
Butanol	32·61	27·27	43·49	21·25	58·81	59·9	60·4
Ethanol	65·22	65·66	44·76	75·26	41·19	12·6	9·0
Final acidity (ml. 0·1 N acid/10 ml.)	7·1	7·9	7·9	7·3	5·9	3·4	2·3
Residual starch (iodine test)	Present	Present	Present	Present	Present	Absent	Absent

* Figures for *Cl. roseum* and *Cl. felsineum* taken from Langlykke *et al.* (1935).

Table 7. *Fermentation of yeast-glucose by* W 45, Cl. roseum* *and* Cl. felsineum* *at* 37°

	W 45		*Cl. roseum* strain 42	*Cl. roseum* strain 43	*Cl. felsineum* strain 41
	1	2			
Glucose fermented	60·4 %	45·9 %	56·5 %	46·5 %	79·6 %
Acidity (ml. 0·1 N acid/10 ml.)	6·4	6·9	9·85	9·05	6·70
	Neutral volatile products, as % of glucose fermented				
Butanol	4·9	3·5	4·6	2·6	14·4
Ethanol	8·7	11·7	3·5	3·1	1·8
Acetone	1·0	0·6	1·5	1·0	4·4
*iso*Propanol	1·8	2·1	—	—	—
Total solvents	16·4	17·9	9·6	6·9	20·6

* Figures for *Cl. roseum* and *Cl. felsineum* taken from Langlykke *et al.* (1935).

Action on pectin. The new chromogen definitely ferments pectin (see Table 2), a characteristic shared by *Cl. roseum* and *Cl. felsineum*. When grown on carrot wedges, the organism causes a marked disintegration of the tissue (Pl. 1 C 2). Definite signs of softening and rotting are observed within 48 hr.

Microscopic examination of the softened tissues reveals that a certain amount of separation of the cells has occurred, but that the cell walls are left intact. On staining with ruthenium red, the stain is in many instances localized in certain places and not evenly distributed around the cell walls. This suggests that the pectic substances of the middle lamella cementing the cells together are attacked by the organism. Potato slopes on the other hand show no signs of disintegration (Pl. 1, C1). It is interesting to compare this with certain inoculation experiments carried out on potato and carrot slopes with strains of *Cl. pectinovorum* (Weizmann & Hellinger, 1940). Two of the strains brought about a slow but perceptible breakdown of the potato tissue, but the carrot slope was left intact.

The ability of the new bacterium to attack pectin is of interest because of the claims made by various investigators that certain species of clostridia are the causal agents of retting; *Cl. felsineum* is known to be a true retting organism.

Table 8. *Comparative measurements of* W45, Cl. roseum *and* Cl. felsineum

	W45 24 hr. on maize (μ.)	*Cl. roseum* 24 hr. on maize* (μ.)	*Cl. felsineum*† (μ.)
Rods:			
Limits of size	**2·6–13·0 × 0·5–0·6**	**3·2–4·3 × 0·7–0·9**	**3·0–5·0 × 0·3–0·4**
Average	**5·6 × 0·54**	**3·9 × 0·8**	
Clostridia:			
Limits of size	**4·5–8·0 × 0·7–1·0**	**5·3–8·8 × 1·1–1·5**	—
Average	**5·8 × 0·82**	**6·7 × 1·4**	
Spores:			
Limits of size	**2·0–2·4 × 0·5–1·1**	**2·5–2·8 × 1·2–1·3**	**2·3 × 1·5–2**
Average	**2·2 × 0·95**	**2·7 × 1·2**	
Spore position	**Sub-terminal**	**Sub-terminal**	**Sub-terminal**

* McCoy & McClung's (1935) measurements.
† Carbone & Tomolato's (1917) measurements.

Pigmentation. The new clostridium generally produces orange-red colonies on yeast glucose agar, but sometimes they may be faintly pink and deepen on standing under anaerobic conditions to a pale orange. The colour is more intense at the centre of the colony, where growth is thicker. On first isolation colonies showed the most intense pigmentation. After numerous subculturings, the colour was less and appeared irregularly. Some colonies develop pigmentation only after some weeks. Pigment production, however, always occurs on maize; on exposure to air, the colour fades in maize mashes. Intermittent subcultivation from maize mash on to yeast glucose agar appears to intensify the pigmentation.

Colonies of *Cl. roseum* on beef peptone glucose agar are stated by McCoy & McClung (1935) to be orange-red; the colour darkens, on exposure to air to almost black, and a purplish pigment diffuses into the agar for several millimeters from the colonies. According to Ruschmann & Bavendamm (1925), colonies of *Cl. felsineum* on carrot juice agar are yellow-red, and the colour darkens on standing in the air to a dirty grey-brown and finally blackish brown. A purplish colour is said to develop from the surface downward in maize cultures of *Cl. roseum* left standing in the air.

Morphology. Too much reliance should not be placed on the value of a comparison of the morphological data summarized in Table 8. The rods of *Cl. roseum* and *Cl. felsineum* appear to be more regular than W45, whereas

W 45 appears to be thinner than *Cl. roseum*. Spores of W 45 are smaller than the spores of *Cl. roseum* and *Cl. felsineum*. Ruschmann & Bavendamm (1925) observed that Carbone & Tomolato's *Cl. felsineum* produced, on potato mash, long threads which broke up into cell-chains of many segments, and also double and single rods. This characteristic has frequently been observed with W 45 on various media.

Sporulation. When subcultured several times at 37°, the new organism loses its ability to sporulate, but quickly recovers it at 30°. This feature does not appear to be a characteristic of *Cl. felsineum* or *Cl. roseum*.

Identity of the organism

There is a certain resemblance between the new chromogen and the two butyl chromogens *Cl. felsineum* and *Cl. roseum*, but it is outweighed by the differences. The new bacterium is a butyric organism, but it is unusual in that it possesses a gelatinase. The organism is, therefore, recognized as a new *Clostridium* for which the name *Cl. aurantibutyricum* is suggested.

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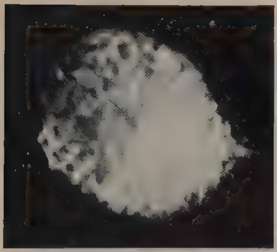
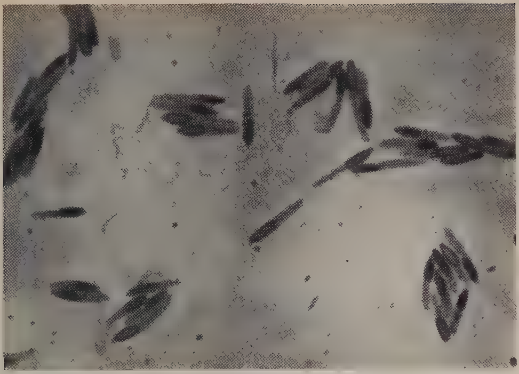


A. Colony of *Cl. aurantibutyricum.* 48 hr. growth on yeast glucose agar under anaerobic conditions at 30° (×23).



B. Clostridial forms from a 6 days old colony of *Cl. aurantibutyricum* on yeast glucose agar at 30° (×1440).

C. 1. Potato wedge, inoculated with *C. aurantibutyricum,* scarcely attacked after 21 days. 2. Inoculated carrot wedge which has disintegrated and fallen into the aqueous solution below the constriction of the tube.

E. HELLINGER—*CL. AURANTIBUTYRICUM* (N.SP.): A PINK BUTYRIC ACID *CLOSTRIDIUM.*
PLATE 1

Some Properties of a Thermolabile Antigen of *Erysipelothrix rhusiopathiae*

By A. W. GLEDHILL
Institute of Animal Pathology, Cambridge

SUMMARY: The production of a thermolabile, somatic antigenic modification by growth of *Erysipelothrix* in media containing serum has been demonstrated *in vitro* by agglutinin-absorption methods and by passive protection of mice against infection. Suspensions produced by growth in serum media have been termed OL-suspensions, to signify their possessing both the thermostable O-antigen, and a labile L-antigen.

OL-suspension stimulated the production of immune sera in rabbits which protected mice fully against infection with the homologous strain and partly against infection with the other strains tested. Such sera contained agglutinins against the thermostable O-antigens, agglutinins against the thermo-labile L-antigen and protective antibodies. The three types of antibodies apparently exist both as separate molecules and united in the same molecule, suggesting that the antigenic groups stimulating them exist united in the same antigenic molecules in the organism. The bearing of this suggested polyspecificity of the antigenic substance of *Erysipelothrix* upon previous work is discussed.

In a previous publication (Gledhill, 1945*a*) it was shown that most strains of *Erysipelothrix* may be assigned by agglutinin-absorption methods to one or another of three groups; the remainder, for convenience, to a fourth group. No thermolabile antigens were found and no qualitative differences between the groups were demonstrated, every strain tested being capable of absorbing the agglutinins from sera prepared against other strains. It was shown subsequently (Gledhill, 1945*b*) that bacterial suspensions, prepared by killing centrifuged cultures grown in glucose broth by heat or merthiolate, induced sera in rabbits which did not protect mice against lethal infection with *Erysipelothrix*, although they gave partial protection, as judged by increased time of survival of the infected mice. On the other hand, living bacteria produced sera that protected mice against lethal infection with the strains used for immunization. It was not easy to absorb the protective antibody from such sera with bacterial suspensions, which suggested that the protective antibody was an antitoxin. This explanation is not, however, consistent with the fact that bacterial suspensions will readily absorb the protective antibody from protective horse sera prepared in a similar way. Moreover, there is no direct evidence that strains of *Erysipelothrix* produce an exotoxin.

The immunizing suspensions were prepared by growing cultures in glucose broth. This paper reports the effect of growth in *serum* broth, which results in the production of bacteria with thermolabile antigens. Furthermore, these bacteria killed with merthiolate produce in rabbits protective sera for mice, comparable in potency with the sera produced by immunization with living cultures. The results suggest that the antigenic properties of *Erysipelothrix* are better explained by the hypothesis that its antigenic groups are present in single antigen molecules, rather than by the hypothesis that each reactive group represents a separate antigen molecule.

Methods

The strains of *E. rhusiopathiae* employed were of high virulence for mice. Suspensions from these strains prepared by centrifuging glucose broth cultures and resuspending the deposit in normal saline containing 1:2000 merthiolate, as previously described, are referred to as O-suspensions. Suspensions prepared by the same technique from cultures in 1% (v/v) horse serum broth, are called OL-suspensions, and usually consisted of a strain that had been subcultured daily in serum broth for about ten days.

Preparation of antisera. To economize antigen, the immunizing dose previously used in rabbits was reduced to approximately a quarter.

As will become evident later, it was important to know that the bacteria were in fact all killed by the merthiolate. This was tested by direct plating, discovered by trial to be *in vitro* the most sensitive method, and by mouse inoculation. In the experiments described below all antigens are merthiolate-killed unless otherwise stated.

Test of antisera. Antisera were tested both for agglutinins and their power to protect mice against infection with a dose of virulent organisms having an opacity equal to that of 100,000 *Bact. coli* per ml., which represents about 2000 minimal lethal doses (M.L.D.). Absorptions were carried out for three days at room temperature. The technique of these tests was described by Gledhill (1945 *a*, *b*).

EXPERIMENTAL

The induction of an antigenic modification by growth in serum broth

The antigenic modification induced by growth in serum broth is demonstrable by agglutinin-absorption and by mouse protection tests.

(*a*) *Demonstration of antigenic modification by absorption of agglutinin.* A protective antiserum (A 73) prepared against *viable* group I strain 1224 was absorbed with the homologous O-suspension. The resulting absorbed serum was free from O-agglutinins, but agglutinated OL-suspensions. An antiserum (984) was prepared against the merthiolate-killed OL-suspension of the group II strain EW 2 and absorbed with the homologous O-suspension. This absorbed serum, free from O-agglutinins, also contained L-agglutinins (Table 1). It is to be noted that the OL-suspension of strain 1224 was not agglutinated by the absorbed serum 984. However, another serum (987), prepared in a different rabbit against the OL-suspension of the same strain EW 2 and absorbed with the homologous O-suspension as in the case of serum 984, was found to agglutinate the OL-suspension of 1224, the titres of this absorbed serum against the OL-suspensions of EW 2 and 1224 being 1:640 and 1:160 respectively. The inability of absorbed serum 984 to agglutinate OL-suspension of strain

1224 suggests that the modification induced by growth in serum media may be complex (see below).

(*b*) *Demonstration of antigenic modification by mouse protection experiments.* The above observations suggested that sera prepared against merthiolate-killed OL-suspensions might be protective for mice in the way that sera against viable cultures are protective. A serum (904) prepared against an OL-suspension of strain EW 2 was titrated in mice infected 24 hr. later with the usual dose of the virulent homologous strain. A dose of 0·1 ml. of serum protected all of six mice, and a dose of 0·01 ml. only two out of six mice. Four control mice died in an average time of 2·3 days and six mice given 0·1 ml. of a serum prepared against the O-suspension of EW 2 died in an average time of 5·3 days. In further experiments, doses of 0·1 ml. of sera 984 and 987 prepared against different batches of EW 2 OL-suspension were completely protective. The protective power of these sera were not, therefore, inferior to those described previously prepared against living bacteria. The validity, however, of the conclusion that OL-suspensions stimulate the production of effective immune sera in rabbits rests on the assurance that no viable organisms remained in the suspensions employed. For, if even a few cells remained viable, the efficacy of the resultant immune sera could presumably be attributed to these. The methods of sterility testing were rigorous enough to exclude this possibility. Moreover, O-suspensions killed in the same way have never induced protective antibody in the rabbit (Gledhill, 1945 *b*).

Table 1. *Titres with OL-suspensions of antiserum prepared* (*a*) *against living* 1224 *and* (*b*) *against killed OL-suspensions of EW* 2, *each absorbed with its homologous O-antigen*

Antigen	Group	(*a*) Serum A 73	(*b*) Serum 984
EW 6	I	1:80	1:160
1224	I	1:160	—
Ru	I	1:160	1:160
AE 5	I?	1:40	1:160
EW 2	II	1:80	1:160
EW 11	III	1:80	1:160
V	III	1:160	1:160
MEW 22	IV	1:80	1:160

Neither serum agglutinated the corresponding O-suspensions.

The lability of the antigenic modification

(*a*) *Thermolability.* It seemed possible that the antigenic modification might be thermolabile, in view of the well-known difficulty of immunizing pigs with heat-killed vaccines prepared in various ways. The thermolability of the new antigen was determined by following the consequences of heat treatment upon its agglutinability by L-antisera and upon its power to induce mouse-protective antibodies in the rabbit.

The agglutinating titre of serum A 73, prepared against viable group I strain 1224 and absorbed with homologous O-suspension, was determined for five portions of a single merthiolate-killed OL-suspension of EW2 maintained for ½ hr. at 55°, 65°, 75°, 90° and 100° respectively. None of the heated suspensions was agglutinated at the lowest dilution tested (1:20), whereas the titre with unheated suspension was 1:80.

Similarly treated portions of EW2 OL-suspension were used to immunize rabbits. The protective effect of the resulting sera in mice shows that the new antigen is completely destroyed in ½ hr. at 75° and largely destroyed at 55° (Table 2).

Table 2. *The protective power of antisera prepared against merthiolate-killed OL-suspensions of EW 2 heated to various temperatures*

Rabbit no.	Treatment of immunizing suspension	Dose of antiserum (ml.)	Death-rate (6 mice)	Average time to death (days)
984	Unheated	0·1	0/6	—
984	Unheated	0·05	2/6	14·0
980	Heated to 55° for 30 min.	0·1	3/6	5·0
980	Heated to 55° for 30 min.	0·05	6/6	6·0
906	Heated to 65° for 30 min.	0·1	3/6	6·7
862	Heated to 75° for 30 min.	0·1	6/6	6·2

(*b*) *Ethanol resistance.* A batch of growth was treated for 48 hr. at room temperature with ethanol by the method of Henderson (1937). The ethanol was removed by centrifugation and the suspension, after washing three times in saline, was used to immunize a rabbit. The serum in a dose of 0·3 ml. completely protected six mice against the usual infecting dose of organisms. With a dose of 0·1 ml. two out of six mice survived, the four dying in an average time of 7·0 days as compared with 3·3 days for the controls. On the other hand, 0·1 ml. of serum prepared by immunizing another rabbit with merthiolate-killed antigen obtained from the same batch of growth failed to protect two out of six mice. It appears that the antigen stimulating the production of protective antibodies in rabbits was not destroyed by the ethanol treatment.

It may be concluded from these experiments that the growth of *Erysipelothrix* in the presence of horse serum induces a somatic, thermolabile, antigenic modification, resistant to ethanol. The somatic Vi-antigen, described by Felix & Pitt (1934) in virulent strains of the typhoid group of bacteria, is thermolabile and ethanol resistant. Whether the antigenic modification of *Erysipelothrix* by growth in serum resembles the Vi-antigen depends on the definition of Vi-antigens. The antigenic modification, as somatic and thermolabile, accords with the classical notion of Vi-antigen. On the other hand, the antigenic modification of *Erysipelothrix* is not strictly associated with virulence since a number of strains of low virulence—for instance 1224 and EW11 of Table 1—produce it, although several rapid transfers in serum media may be necessary before its presence can be demonstrated. Strains in the partially rough phase do not appear to produce it. There is, however, no doubt that a smooth strain devoid of L-antigen as a result of growth in broth without serum, may be induced to produce it by growth in the presence of serum. Hence, the presence of serum does not serve merely to hinder or prevent an irreversible antigenic degradation consequent upon maintaining cultures *in vitro*. In fact, the antigenic modification is induced by the medium selected for growth. Whether the loss of the capacity to produce the antigenic modification is reversible is, however, a different question.

The specificity of the antigenic modification

In the experiments so far described, only sera against OL-suspensions of the group II strain EW2 were used. The agglutination tests with absorbed sera shown in Table 1 suggest that the antigenic modification is not strain specific.

Table 3. *The cross-protection afforded by antisera prepared against OL-suspensions*

The dose of serum was 0·1 ml. per mouse.
All 15 controls died in average times of 3·2 days for EW2 and 3·0 days for each of Ru and V.

Protective serum				
Rabbit no.	Prepared against	Infecting strain	Death-rate (5 mice)	Average time to death (days)
984	EW2 (II)	Ru (I)	1/5	5·0
		EW2 (II)	0/5	—
		V (III)	2/5	6·5
A26	Ru (I)	Ru (I)	0/5	—
		EW2 (II)	3/5	7·8
		V (III)	1/5	10·0
A5	V (III)	Ru (I)	1/5	10·0
		EW2 (II)	2/5	10·5
		V (III)	0/5	—

It is to be expected that the protective power of such sera would likewise be species specific, especially since sera against viable cultures are not strain specific. To test this point sera were made against the OL-suspensions of the group I strain Ru and the group III strain V, in addition to serum 984 against the group II strain EW2. Strains Ru and V were chosen because they were highly virulent for mice and therefore suitable for infecting the test mice and, with EW2, represented the heat-stable antigens of all three groups. The protective power of the sera is shown in Table 3. It is clear that the cross-protection by these sera is inferior to the protection by sera against the homologous strain and especially so in the case of the serum prepared against strain Ru. Nevertheless, that there is some degree of cross-protection is evident when it is recalled that the infecting dose was about 2000 M.L.D. and that mice receiving anti-O sera do not survive infection with such a dose of virulent organisms. For example, of forty-five mice injected with sera of this kind, forty-three died in an average time of 4·7 days, the two survivors being from an experiment in which the virulence of the infecting organisms was somewhat reduced, judged by the time of survival of the controls. This imperfection of the strain specificity of the antigenic modification again suggests that it may be complex.

The complexity of the antigenic modification

The following experiments suggest that the antigen is dual in that it stimulates in rabbits agglutinins and protective antibodies, which can occur in antisera as separate particles.

Serum 984, prepared against the OL-suspension of strain EW2 (II) and absorbed with the homologous O-suspension, agglutinates OL-suspensions but not O-suspensions of other strains (Table 1), but was unable to protect mice against the usual dose of homologous infecting organisms. On the other hand, absorbed serum 987, prepared in the same way as 984, against the same strain EW2, not only agglutinated OL-suspensions but gave a significant measure of protection to mice. Serum 863, prepared against *viable* EW2 and absorbed with the homologous O-suspension, failed to agglutinate OL-suspensions, yet gave good protection to mice, and serum A273 against viable 1224 did not protect mice after absorption with the homologous O strain, although it remained capable of agglutinating OL-suspensions (Table 1). The details of these experiments are shown in Table 4. Moreover, when the strain EW2 is grown in rabbit or pig serum broth, the growth, like that in horse serum broth, is agglutinated by absorbed OL-antisera. Yet it did not induce protective antibodies for mice on injection in the rabbit (Table 5). This result also suggests that the modification induced by growth in serum media is complex. It is of interest that the Vi-antigen of *Salm. typhi* appears to be similarly complex in that antisera to formolized broth cultures of Vi typhoid bacilli agglutinate virulent typhoid bacilli although conferring very feeble protective powers upon mice (Felix & Bhatnagar, 1935). Nevertheless, antisera to living virulent typhoid bacilli contain both agglutinating and protective antibodies.

Table 4. *Agglutinin content and protective power of antisera absorbed with O-suspensions*

The dose of serum was 0·1 ml. per mouse.
All the 16 controls died in an average time of 3·4 days.

Rabbit no.	Serum		Agglutinating titre of absorbed serum against EW2 OL-suspension	Death-rate (5 mice)	Average time to death (days)
	Prepared against	Absorbed with			
984	EW2 OL-antigen	EW2 O-antigen	1:160	5/5	3·6
987	EW2 OL-antigen	EW2 O-antigen	1:640	2/5	10·5
863	EW2 viable	EW2 O-antigen	>1:10	0/6	—
A73	1224 viable	1224 O-antigen	1:160	4/4	8·0

Since protective antibodies appear to be distinct from specific agglutinins for OL-suspensions, the fact that the agglutinogen is attached to the organisms does not entail that the antigen inducing the protective antibody is so attached, it might exist in solution in the suspending fluid. However, the bulk of the antigen appears to be attached to the bacterial bodies, for ethanol-treated, washed bacteria will induce protective antibodies in a rabbit. This hypothesis has been confirmed in a number of experiments in which the bacteria were treated chemically with a view to liberating the antigen; the bulk of the antigen remained attached to the killed organisms, as shown by the superior antigenicity of the treated organisms as compared with the supernatant fluid from the treated suspension.

Table 5. *Protective power of antisera prepared against suspensions grown in media containing horse, rabbit and pig serum*

The dose of serum was 0·1 ml. per mouse.
All 15 controls died in an average time of 3·8 days.
The suspensions used for preparing the three sera were all agglutinated to the same titre (1 : 160) by serum 984 absorbed with the homologous O-antigen.

Rabitt no.	Protective serum against antigen prepared by growth in	Death-rate (5 mice)	Average time to death (days)
984	1 % horse serum	0/5	—
A6	1 % rabbit serum	5/5	5·6
A16	1 % pig serum	5/5	10·6

DISCUSSION

Reasons have been advanced for assuming the complexity of the thermolabile somatic antigenic modification of *Erysipelothrix rhusiopathiae* induced by growth in serum media. The immunological data upon which this assumption is based are summarized in Table 6. From the absorption experiments it would appear that the thermostable O-antigens are sometimes capable of absorbing the majority of the L-agglutinins (serum 863), and are sometimes capable of absorbing the majority of the protective antibodies (sera 984 and A78). If it is assumed that non-specific absorption did not occur, then sometimes most of the agglutinins for OL-suspensions appear to be associated with

Table 6. *Summary of the results of the immunological tests*

			Residual antibody effect		
				Agglutinin	
Serum	Nature of immunizing suspension	Absorbing suspension	Protective	L	O
Previous paper	Living cells	Nil	+	nt	+
		O	+	nt	−
Previous paper	O	Nil	− or ±	nt	+
A78	Living cells	Nil	+	+	+
		O	−	+	−
984	OL	Nil	+	+	+
		O	−	+	−
987	OL	Nil	+	+	+
		O	+	+	−
863	Living cells	Nil	+	+	+
		O	+	−	−

nt = not tested.

the O-agglutinins and at others it is protective antibodies that appear to be associated with them. On other occasions neither the agglutinins for OL-suspensions nor the protective antibodies are associated with the O-agglutinins. It is generally believed (see e.g. Dean, Taylor & Adair, 1935), that the components of a mixture of antigens stimulate antibody production independently of each other. On the other hand, following the work of Heidelberger & Kendal (1934), it has been suggested that a single antigen molecule, possessing more than one kind of reactive group, can give rise to antibody molecules specific for one group only, for some, or, for all the groups (Marrack, 1938). Generally speaking, antibody molecules of all these kinds would be present, the quantitative distribution of each being subject to wide variation. Certainly prolonged immunization tends to the production of antibodies of less sharp specificity (see Burnet, 1941). It is not clear, however, whether the explanation of this observation is that antibodies with more than one distinct specificity tend to be produced by prolonged immunization or that for other reasons a single specificity tends to be broadened. If the former, the results of the experiments described suggest that the antigenic group stimulating agglutinins against OL-suspension, the antigenic group stimulating protective antibodies and the thermostable antigenic groups are all united in single antigen molecules upon *Erysipelothrix* organisms. In the light of this hypothesis, the fact that protective antibody is easily absorbed by O-suspensions from horse antisera to *viable* bacteria and yet not completely absorbable from rabbit antisera to *viable* bacteria (see Table 5) can be explained as follows. The two sera differ in the concentration of protective antibody molecules as compared with that of dispecific antibody molecules capable of reacting with the thermostable O-antigens and of conferring protection. In horse immune sera, the polyspecific antibodies presumably predominate, monospecific antibodies predominating in rabbit immune sera. The reasons for the difference in concentration of the two kinds of antibody in horse and rabbit antisera are more obscure. Horses are not so easily hyperimmunized against *E. rhusiopathiae* as rabbits are, and require a more prolonged course of inoculation, with the result that polyspecific antibodies are likely to be produced more abundantly. Alternatively, it might be supposed that growth in serum medium produces a thermolabile antigenic modification without involving the production of any new antigenic groups. Antisera against such a modified antigen would contain some antibodies of sufficiently wide reactivity both to confer protection upon mice and agglutinate O-suspensions; others would confer protection and agglutinate OL-suspensions, not O-suspensions; others, again, would only confer protection. According to this hypothesis, the fact that the protective power of horse immune sera can be absorbed by O-suspensions, whereas that of rabbits cannot, would result if horse immune sera contained a greater proportion of antibodies of wide reactivity than do rabbit sera. No discrimination between these hypotheses is possible other than by determining the differences of chemical structure between purified O- and L-antigens. In the absence of any real basis for such a discrimination, the former hypothesis is to be preferred in that it justifies the postulation of thermolabile L-antigenic groups whereas the latter hypothesis would require that OL-suspensions should differ from O-suspensions in an unknown manner, leading to difficulties in exposition. For example, the notion of bacterial growths in serum media as containing O + L antigenic groups would be invalid. Both hypotheses, however, imply that the production in rabbits of all the types of antibodies under discussion is not stimulated by a number of separate antigenic substances in each organism.

In regard to the difficulty previously experienced of completely absorbing with living suspensions the antibodies in rabbit sera prepared against such suspensions, it is to be noted that the absorbing suspensions were obtained from glucose broth cultures, and would not possess L-antigen. The question whether the thermostable O-antigens investigated previously occur in the organism as separate antigen molecules or whether they are associated with other antigens on the same molecule, does not modify the conclusions previously reached about the relations between the O-antigens of different strains of *Erysipelothrix*, except perhaps verbally.

The nature and causes of the antigenic modification offers an interesting field for inquiry. Does serum globulin itself, or some other constituent, induce the modification? It is noteworthy that the modifications induced by rabbit and pig sera differ from those induced by horse serum in their apparent inability to produce protective antibodies. The difference resembles the differences of virulence of organisms induced by *passage* in different species of animals. Hence, a knowledge of the nature of the serum-induced modification might have some bearing upon the wider question of the variations in virulence of strains of *E. rhusiopathiae*. The observation that viable organisms, grown in glucose broth, centrifuged and suspended in serum do not acquire the L-antigenic modification, and the fact that the antigenic modification is best produced by several rapid subcultures in serum media, suggest that active multiplication is necessary for the production of the L-antigen. Finally, there is the question as to whether the loss of the capacity to modify when grown in serum can be correlated with the S → R change and whether such loss is reversible. The possible bearing of the answers to these questions upon the epidemiology of the disease in pigs and other animals is obvious.

From the more immediate practical standpoint, the possibility that killed OL-suspensions might prove successful in the active immunization of pigs against swine erysipelas is of some importance, since such vaccines would be safer and more economical than the simultaneous administration of living bacilli and immune serum used at present.

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The Preparation of Microtools for the Micromanipulator

By A. C. THAYSEN and A. R. MORRIS
Colonial Microbiological Research Institute, Trinidad, British West Indies

SUMMARY: Tools required for the more delicate processes of micromanipulation are not commercially available and existing equipment for their making is expensive. Some cheaper equipment is described, comprising a gas forge and an electric forge which together suffice to prepare the tools needed for isolating single cells, for injecting and dissecting living tissue and living cells, and for determining their electrical potential.

A simple hanging-drop micromanipulation chamber is described.

Though a considerable amount of information is available on the technique of micromanipulation, notably in American and German publications, only those of Schouten (1934) and de Fonbrune (1937) give a detailed account of the equipment needed for making the tools used in the more delicate processes of micromanipulation, the dissection of cell structures and the injection of liquids into living microscopic cells. As neither of these publications is readily accessible, our observations on this subject may be of interest. Our methods for making microtools are based essentially on the work of both Schouten and de Fonbrune, but are simplified by the use of a more standardized equipment, which should be cheaper to construct than the forge designed by de Fonbrune. The design of one of the most frequently used manipulators is based on the work of Chambers, whose paper appeared in 1922. Schouten's work on micromanipulation, to the perfection of which he has devoted most of his scientific life, was begun as early as 1899, making him the undoubted originator of this fascinating technique, which to-day has acquired great importance in the exploration of the genetic behaviour of micro-organisms.

The preparation of the microtools requires two forges, one heated by gas, the other electrically.

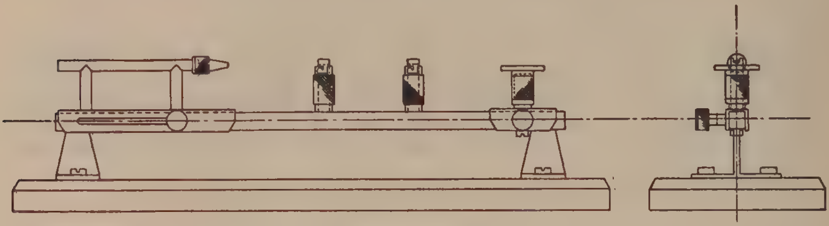
The construction of the gas forge

The gas forge consists of a bar or bed 11½ in. long by ⅜ in. square, mounted on supports, upon which are placed two sliding members and two fixed supports. It is made of brass and where convenient, all joints are silver soldered.

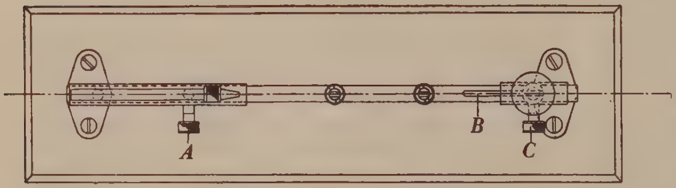
Text-figs. 1–5 show the general arrangements of the forge; the enlarged views show the construction of the sliding members and the supports.

The spring-loaded head (Text-fig. 3) is the most important part of the instrument. It consists of a tubular section 3 in. long housing the spring and stem for applying tension to the glass tube. The whole head is movable along the bar or bed to the extent allowed by the slot *B* (Text-fig. 3) and is fixed in its required position by the knurled screw *A* (Text-fig. 2).

The spring is approximately $3\frac{1}{2}$ in. long and consists of 60 turns of no. 11 m.w.g. wire wound on a $\frac{7}{64}$ in. former. The stem is a $\frac{1}{8}$ in. diameter rod with a $\frac{3}{16}$ in. diameter head, screwed at the opposite end to carry the chuck *A* (Text-fig. 3). At its tip the chuck is provided with a shallow counter-sink to centre the glass tube when it is attached with sealing wax.



Text-fig. 1



Common to Figs. 1 and 2.

Text-fig. 2

The two fixed supports (Text-fig. 4) are secured to the bar by counter-sunk screws and are in two parts, the fixed central pillars, and the knurled outer sleeves. The central pillars have a U-shaped groove at the top of which rests the glass tube. The knurled outer sleeves have a bayonette clip arrangement at their top edge and slide freely on the central pillars. The top edge of the sleeve is located just below the root of the U-shaped groove in the central pillar. When the glass has been placed in the grooves the outer sleeves are lifted and rotated. The glass tube is then engaged in the bayonette clips. The weight of the sleeve is sufficient to steady the tube for further manipulation.

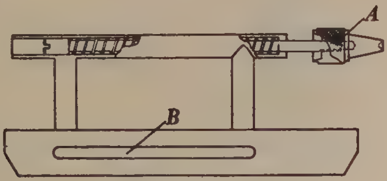
The other slidable member is the adjustable table (Text-fig. 5). Its sliding movement is limited by the length of the slot *B* (Text-fig. 2) cut through the bar, and by the screw which passes through the slot into the base of the table. The table can be fixed where desired by the knurled screw *C* (Text-fig. 2).

Rising or falling adjustment to the table is made by screwing the table up or down on the stem *C* (Text-fig. 5). A very small amount of movement is needed, in fact once the table has been set at the correct height further adjustment is not necessary unless the glass tube is bent or of some special shape.

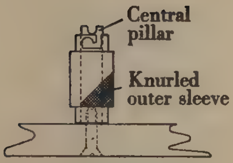
As shown, the table is spring-loaded. This is not absolutely necessary, but it adds greatly to the stability of the table.

The gas microburner

The microburner is a simple burner having as its chief feature a very small jet. Some elaboration has been made in the construction of the jet and has proved worthwhile. The burner is built up in brass and silver soldered; the large diagram (Text-fig. 6) shows the construction in detail. The jet *A* (Text-fig. 6) is screwed into the stem *B* and seats on the conical face *C* to form a seal below the hole *D*. The screw *E*, with the needlepoint, has a groove cut in it to allow the gas to pass up to the valve seat *F* where it is restricted before passing on to the small hole at the tip. This construction provides a means of regulating the size of the flame. The adjustment to the worker's need is soon found. Below the screw *E* is a small plug of cotton-wool to filter the gas and prevent any foreign particles entering and blocking the jet.

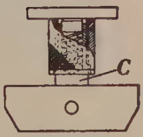


Text-fig. 3



Text-fig. 4

Common to Figs. 3–5.



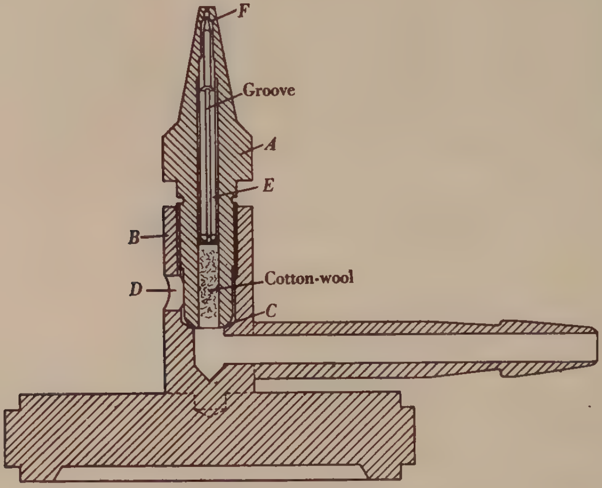
Text-fig. 5

By unscrewing the jet from the conical face *C* for a few moments, air trapped in the line is allowed to escape by the hole *D*. This makes it possible to light the burner almost immediately on turning on the gas. If this is not done, the time required to get rid of any air through the jet is considerable. It will be noticed that it is not necessary to make provision for primary air with this burner.

The size and general construction of the burner can be made to suit the individual worker, the only detail of importance is the size of the final hole in the jet which should be in the order of 0·01 in.

The gas forge is needed to reduce the diameter of the commercially available glass tubing as a first step in the fashioning of microtools. The original diameter of this tubing is governed by the size of the holder of the micromanipulator (Pl. 1, fig. 1) into which the glass tubing carrying the final microtool has eventually to be fitted.

When the gas-heated forge is used, a length of commercial glass tubing, usually of soft soda glass and of a diameter suitable to be held in the manipulator holder, is placed on the two central pillars of the forge. One or both of these is then locked. The two ends of the tubing are next sealed with sealing wax to the spring-loaded head and the surface of the table. When the sealing wax has solidified, tension is brought to bear on the glass tubing by moving the table to the right and clamping it there by means of the appropriate screw. This movement contracts the spring within the head. This, in its turn, can be moved in a horizontal direction to increase the tension on the glass tubing if needed.



Text-fig. 6. Cross-section of gas microburner. × 1·4.

The microburner is now applied to the glass tubing, between the head and the first pillar and the glass is softened until the pull of the contracted spring in the head overcomes the tension of the glass. When this point has been reached the recoiling spring elongates the glass tubing to an extent which depends on the recoil of the spring and on the temperature of the glass.

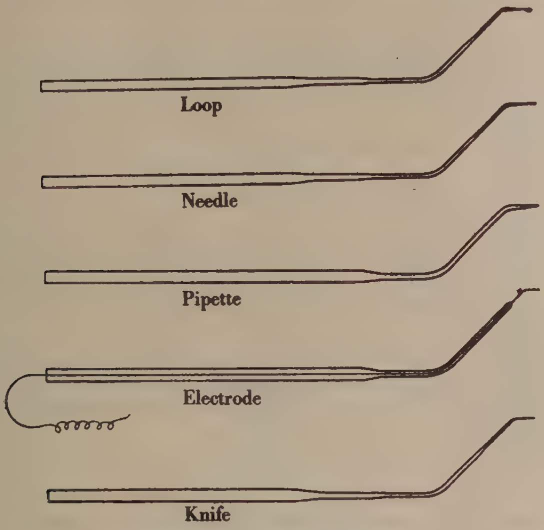
The glass tubing is next cut with a pair of scissors at its narrowest point and lifted from the table. The end covered with sealing wax is broken off and the fracture trimmed in the microburner. When cool the tube is inserted and fixed in the manipulator holder and is then ready for the making of the more delicate microtools.

An illustration of some of these tools is given in Text-fig. 7. A detailed description of making these tools is given below. Coarser pipettes and tools for fracturing sporangia can be made wholly in the gas forge; the more delicate microtools only in the electrical forge.

The electrical forge

The component parts of the electrical forge are shown in Text-fig. 8.

The platinum filament, inserted in an insulated holder, is designed for clamping to one of the two manipulator hands, since the finer microtools are made entirely under microscopic control. The heater is wired to a 12 V. transformer, and to a resistance by which the current to the filament is regulated. The insulated holder consists of a brass rod on which is mounted an ebonite block *A*, and an ebonite guide *B*, both of which are drilled to accommodate the conductor rods of the platinum filament. The holes in the guide block *B* allow the conductor rods, which are of 16 s.w.g. copper wire, to slide through freely and pass on through the holes in the terminal block *A*. In the terminal block the holes are of such clearance that when the terminal studs 1 and 2 are locked, and the knurled clamping screws 3 are adjusted, the conductor rods are held between them. The glass tube *C* protects the copper conductor from accidental contact with the manipulator. The platinum filament is easily constructed, very robust, and convenient to handle. The two 16 s.w.g. copper conductor rods *D* are flattened at one end to about half their thickness for a distance of approximately $\frac{3}{16}$ in. and clipped to neat spear points. They are inserted into the holes drilled in the small ebonite block 5 and the flats of the spear points set in the same plane and at the requisite distance from the block. A small blowpipe flame is carefully applied to the copper rods at a safe distance from the ebonite. The heat conducted along the copper will soon cause the ebonite round the copper to melt and at that point the heat is carefully quenched in water. When cold the copper rods will be found to be securely anchored in the ebonite which forms a very convenient grip when handling the completed filaments.

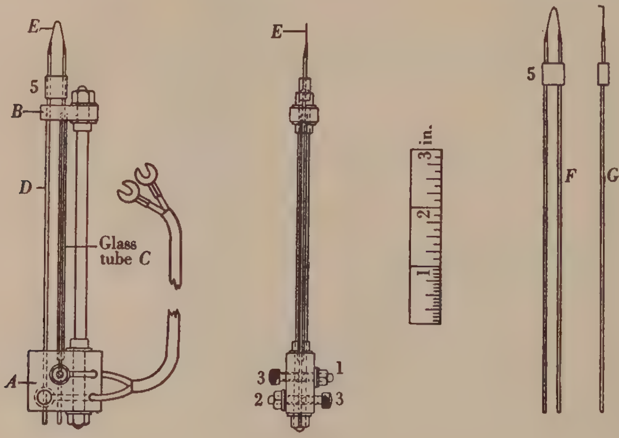


Text-fig. 7. Glass microtools.

All that now remains to be done is to solder the platinum wire filament *E* on to the conductors. The filament is 0·20 mm. wire, 19 mm. long; it is attached with ordinary tinman's solder. Although the solder does not readily wet the platinum, a sound connexion is easily achieved. Work can be done with the filament at almost white heat without fear of melting the solder since the heavy gauge of the conductors quickly dissipates the heat.

Considerable skill is required to weld the tip on the filament to form the anvil illustrated in Text-fig. 8, *F* and *G*. After the filaments have been constructed the tip is hammer welded as follows:

A piece of platinum wire 1 in. long is soft soldered to a 3 in. length of 16 s.w.g. copper wire to give it some stability and to make manipulation easier. The completed filament and the 1 in. length of platinum wire are set up for welding on a steel plate, preferably hardened and polished. When the filament is laid on the steel plate the ebonite block will prevent the platinum from touching the surface of the steel. As the distance between the platinum wire and the plate is too great it should be reduced to about $\frac{1}{32}$ in. by gently bending the wire downwards. The tip of the 1 in. length of platinum wire is now carefully laid on the top of the platinum filament at the point where the weld is to be made, noting that the $\frac{1}{32}$ in. gap between platinum wire and steel plate is retained. Small metal blocks may be used to hold the parts in their correct position during the subsequent welding operation.



Text-fig. 8

A small hot blowpipe flame is next directed on to the point where the weld is to be made and, the correct welding temperature having been reached, a carefully directed tap with a small hammerpoint completes the union. All that now remains is to snip off the anvil tip at the correct length, approximately 3 mm.; the remainder of the 1 in. length of platinum wire can be used to produce more anvils.

When the electrical forge is to be used, its heater is first clamped into position on one of the manipulator's two hands, for right handed workers preferably in the right hand of the manipulator.

In the left hand is inserted a holder with drawn out glass tubing. The tip of the glass tubing and of the platinum filament are next centred opposite each other and directly under the low objective of the microscope so that they may be viewed simultaneously while the tool is being shaped. A magnification of approximately ×100 is convenient for the fashioning of most tools.

The filament of the heater can be replaced by others of different size or design. They include types which hold a glass or metal bead at their tip and others, anvils, with a short platinum rod welded to their tip (Text-fig. 8, *F* and *G*). The latter serve as anvils in the making of microloops and in moulding the base, or handle, of most microtools used in hanging-drop preparations. Metal and glass beads can be fused to the filament by heating it to a bright glow and touching a thin metal or glass rod with it. The glass or metal will melt and collect as a droplet at the tip of the filament. The ensemble for making microtools is set out in Pl. 1, fig. 2.

Microneedles

A platinum filament, holding a glass bead at its tip, is inserted into the heater and centred opposite the drawn glass tubing within the low-power optical system.

By adjusting the resistance of the electrical forge the glass bead is softened to the consistency of thick treacle. The glass tubing in the left hand of the manipulator is now moved towards the molten glass bead and fused to it.

The two manipulator hands are next moved away from each other in a horizontal direction. The pace of this movement, which can be learned only by experience, must be such that the glass rod withdrawn from the glass bead by the pulling movement is maintained at a uniform diameter. The temperatures of the bead and the rate of pull will govern the diameter. When the right length of glass rod has been withdrawn, the heat is cut off. The pull exercised on the rod by contraction of the cooling glass bead is normally sufficient to fracture the glass rod at its point of least resistance, that is nearest the glass bead, if care has been taken to draw the glass rod slightly thinner at this point. When the cooling glass bead does not exercise sufficient pull, the break is made by an extra pull from the two manipulator hands. The resulting rod serves as the handle of the needle. The needle point is made by remelting the glass bead and fusing the handle and the bead. A sharp pull of the manipulator hands draws the handle into the needle point. It is now ready for use if a straight instrument is required. For work on cells suspended in a hanging drop and sealed from evaporation by an overlying layer of liquid paraffin, the handle of the needle must be bent to an angle of approximately 45°, followed by a deflection of the needle itself almost back to the horizontal (Text-fig. 7).

An anvil is inserted in the heater in place of the U-shaped platinum filament and the heater clamped in the right manipulator hand in such a way that the upright piece of platinum wire—the actual anvil—is centred in the microscopic field, just below the handle of the needle. Its top should be some 5μ. higher in the optical field than the handle. The anvil is heated to a temperature high enough to soften the glass of the handle without melting it, and is brought carefully into contact with it. The anvil should be sufficiently sturdy to withstand deformation when pressed against the softening glass handle. The required pressure is obtained by moving the screw of the right manipulator hand governing the optical upward movement and at the same time turning the screw of this hand responsible for the optical forward and backward thrust. As a result the anvil will slide slowly forward and backward along the lower side of the handle, gradually warming and softening it. At the same time the pressure of the anvil exercised on the handle will force the softening handle upwards and out of the straight position. When the handle is correctly bent the anvil is moved from below the handle to above the base of the needle point and is brought into contact with this side of the needle to bend it back towards the horizontal. The procedure now is exactly the same as before except that greater care must be taken in the application of heat and pressure to the thinner needle.

Microloops

A handle is made as described for needles. The tip of this handle is fused to the glass bead to make a second, shorter and thinner handle. This should have a length of 40 or 50μ.; it is broken off from the bead by switching off the current.

An anvil is now centred in the microscopic field immediately opposite the tip of this rod. The top of the anvil should rise no more than 1μ. above the optical height of the short rod. The anvil is gently heated and the short glass rod thrust forward against it by a rapid movement of the appropriate left manipulator hand, until it hits the anvil. If the latter has been sufficiently heated, but not overheated, it will be found that the tip of the short rod bends slightly upwards or sidewards. By repeating the forward thrust of the left manipulator hand, the tip of the short rod gradually shapes itself into a loop, the diameter of which is governed by the heat applied to the anvil and by the vigour of the thrust. Once the loop has been closed, the holder in the left manipulator hand is rotated until the loop is viewed sideways and the anvil is moved below the thicker handle where it is used to bend the handle as already described for microneedles.

Microknives

The procedure for microknives is the same as that for microloops, up to and including the drawing out of a thin glass rod at the tip of the handle. This rod should not be more than 12–20μ. long. The two bends in handle and thinner rod are next made with the anvil.

The holder of the glass tubing is then rotated in its clamp until the thin glass rod is viewed horizontally in the optical field. The anvil is replaced by a filament with a glass bead and this is fused to the thin glass rod. By a sharp pull the thin glass rod is separated from the molten bead, leaving at its tip a short and exceedingly fine rodlet. The diameter of this rodlet, which should not exceed 3μ., is usually less than $0{\cdot}1\mu$. at its end point, and is sharp enough to cut the cell membrane of yeasts and bacteria.

Micropipettes

Micropipettes are drawn out to the desired diameter in the gas forge by moving the adjustable table as far to the right as the contractable spring will allow, and by heating a wider area of the glass tubing than usual. A hot flame is usually required. Recoil of the spring draws out the pipette. The tip of the glass tubing is severed from the micropipette, which is next shaped in the electrical forge. The holder with the straight pipette is clamped in the left manipulator hand and the heater with a U-shaped filament in the right. Both pipette and filament are now centred in the microscopic field, the heater well below the optical plane of the pipette and some 40 μ. to the left of its tip. The filament is heated sufficiently to soften the glass of the pipette at the desired point and to allow the last 40μ. of the pipette to drop downwards until the desired angle is formed. Then the current is cut and the pipette raised within the optical field by rotating its holder, until the bent part is viewed in the optical field in a more or less vertical position.

The filament is now moved below the stem of the pipette some 80μ. behind the bend and some 40μ. below it, and heated sufficiently to soften the glass of the pipette. The softened tip of the pipette is allowed to sink towards the heater until it is almost parallel with the stem when the current is cut.

Micro-electrodes

For making a micro-electrode a fine gauge copper or platinum wire of approximately 0·1 mm. diameter is first passed through the aperture of a standard size glass tube, which is then sealed to the head and the table of the gas forge. The wire at this end should be long enough to allow for the extension of the glass tubing during drawing and to leave enough for an electrical connexion.

When the glass tubing and the wire are inserted in the gas forge and tension applied by sliding the table to the right, the glass is heated in the microburner and drawn out in the usual manner. Instead of cutting the head end of the drawn tubing with scissors, as with other microtools, the whole of the glass tube is lifted from the forge, and the glass at the constricted end broken by gentle tapping with a pocket knife. The fragments of glass are removed from the exposed wire, the latter firmly sealed to the remaining tip of the glass tubing and all but the last 80 to 100μ. of the exposed wire cut away.

The electrode is now inserted into the left-hand manipulator holder and optically centred opposite a U-shaped filament heater. At the tip of this filament has been fused a bead of tin or solder.

When the electrode and the filament have been centred, the metal bead is melted and the wire fused to it. By maintaining the metal bead at a suitable temperature and by withdrawing the end of the electrode from it in a horizontal direction a metal rod is formed at the end of the wire, which can be drawn out into a point fine enough to penetrate a living cell.

The bending of the wire and its point into shape for work in the hanging drop is done without the application of heat with the anvil as described for making microloops.

It remains to be added that, before a glass microtool is used, it should be sterilized by dipping into concentrated sulphuric acid, rinsing in sterile distilled water, immediately transferring to concentrated ammonia, specific gravity 0·880, and again washing in sterile distilled water.

Hanging-drop chamber

Though not strictly a microtool it is appropriate to describe the type of chamber used by one of us (A. C. T.) for work in hanging drops. It differs in several respects from the chambers usually recommended and offers greater freedom of movement.

The chamber consists of a microscope slide 3 × 1 in. to the centre of which is cemented an aluminium cylinder, 20 mm. high and 20 mm. in diameter. The metal is 3 mm. thick. Three slits are cut in the upper rim of the cylinder to a depth of 15 mm.; one slit is 12 mm. wide and two, facing each other at the side of the wider slit, are 8 mm. in width.

The base of the cylinder is joined to the microscope slide with sealing wax or paraffin wax and its top edge covered with a thin layer of vaseline.

In use the chamber is inserted into the moving stage of the microscope so that the major slit faces the light source, and the two side slits the two hands of the manipulator. The chamber is centred under the optical system of the microscope, and a microtool in its holder is inserted into each side slit of the chamber and clamped to the left and right hands of the manipulator. The tools are next focused in the centre of the optical field by movements of the manipulator hands. For certain work, such as the isolation of a single cell, only one microtool is required. For other, such as electrode work and the perforation or dissection of a cell, two and sometimes three tools are required. When three tools are needed, the third is inserted through the slit facing the source of light, and an extra manipulator hand will then have to be provided. The height of the chamber makes it difficult to focus the light source on the hanging drop, but sufficient light can be obtained by a 100 c.p. lamp passing through the Abbé condenser to make it possible to work with a 4 mm. objective. Cells of the organism with which it is intended to work are suspended in a hanging drop on a coverslip of appropriate dimensions and thickness.

It is important first to clean the coverslip carefully before use. Before placing the hanging drop, or drops, on the underside of the coverslip a trace of vaseline is rubbed over this surface and as far as possible cleaned off again with a clean cloth, free from fluff. Complete removal of the vaseline is not possible by such treatment, and is not desirable, for sufficient should be left on to prevent the edge of the hanging drop from running. Schouten (1934)

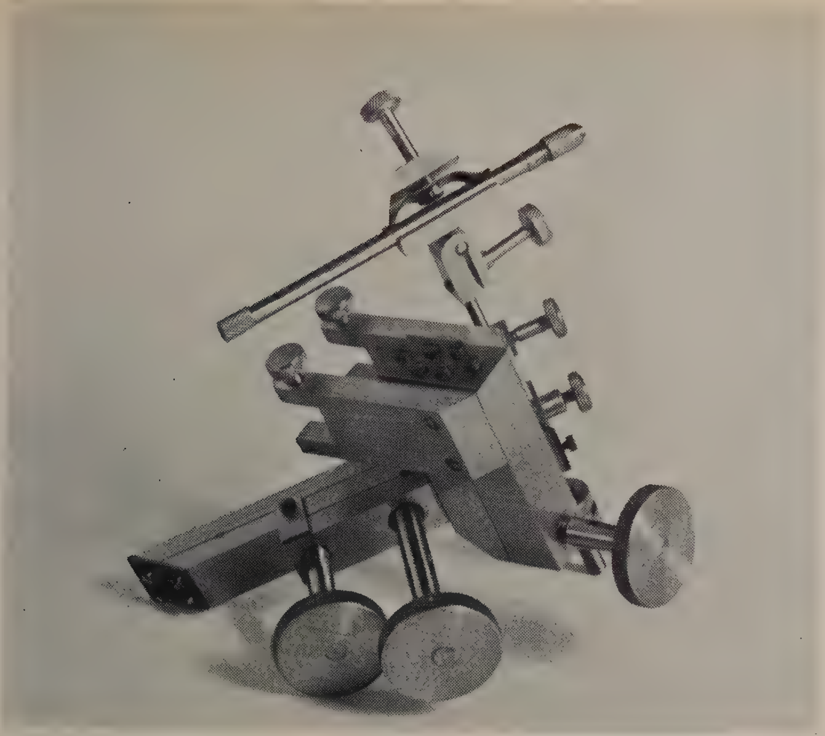


Fig. 1

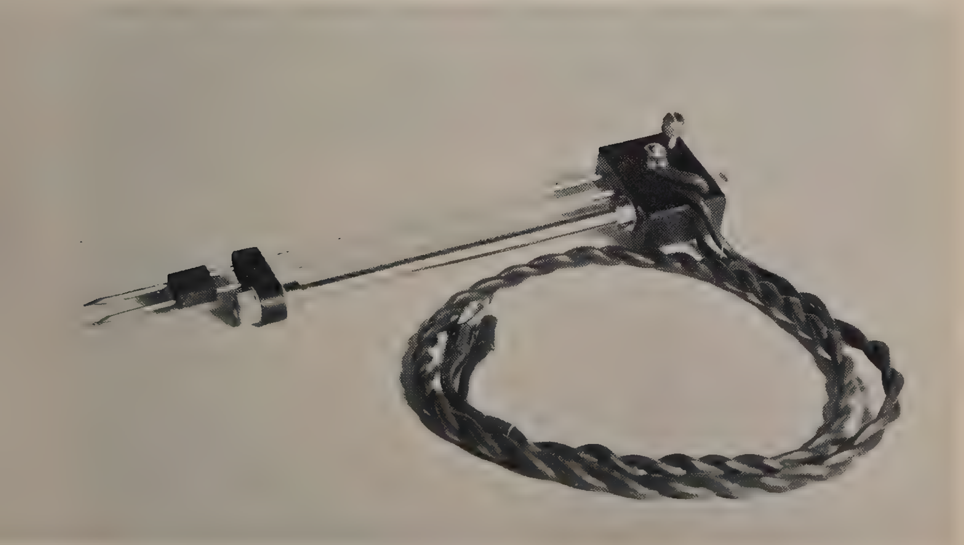


Fig. 2

A. C. THAYSEN AND A. R. MORRIS—THE PREPARATION OF MICROTOOLS FOR THE MICROMANIPULATOR. PLATE 1

recommends the triglyceryl ester of lauric acid for this purpose and states that it is better than vaseline; but, like Barber (1914) the writers have always found vaseline satisfactory.

For the isolation of a single cell at least two minute hanging drops of a suitable nutrient medium are placed on the greased underside of the flamed coverslip, about 0·5 mm. from each other. A very fine platinum loop is used, and each droplet should not exceed 50μ. in diameter. Before the droplets have time to evaporate, a trace of fresh culture of the test organism is transferred to one of the droplets and partially mixed with the medium in the droplet. It is important that parts of the droplet should be free from cells. When the mixing has been too thorough, or the cells introduced too numerous, the isolation of a single cell becomes difficult.

Both or all the droplets are enclosed within a drop of sterile liquid paraffin, using a large platinum loop. The paraffin cover prevents the evaporation of the droplets during work and subsequently protects them from contamination.

For the actual isolation or other manipulation the coverslip with the droplets on its underside is gently pressed on the vaselined top of the chamber. The edge of the droplet containing cells is centred and focused and a microloop plunged into the droplet. When a suitable cell has been caught within the loop the stage of the microscope is moved in the direction of the second droplet, to bring the microloop into the second droplet where it is raised and lowered until the cell is released.

The next stage in the isolation consists in the transfer of the coverslip from the chamber to an ordinary moist chamber at the bottom of which is placed a droplet of sterile water. The slip is sealed to the top of this chamber with vaseline and the chamber then incubated, preferably at room temperature, if this allows the isolated cell to grow. The use of higher temperatures demands careful control of the humidity within the moist chamber and makes it necessary to open the chamber when it is removed from the incubator to a lower temperature for microscopic observation.

As soon as macroscopic growth is visible, the coverslip is lifted from the chamber and the growth transferred in a fine platinum loop to a suitable solid or liquid medium.

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The Routine Examination for Antibiotics Produced by Moulds

By N. G. HEATLEY and FLORA J. PHILPOT
The Sir William Dunn School of Pathology, University of Oxford

SUMMARY: From the existing methods for identifying common antibiotics it is possible to select tests for recognizing one or more of fourteen known antibiotics in crude culture medium. These tests, given in tabular form, are: (1) stability at pH 2·0 and 9·5; (2) ether-water partition at pH 2·0, 6·0–7·0 and 9·0; and (3) relative activity towards specified strains of *Staphylococcus aureus* and *Bacterium coli*. Further tests depend on destruction of penicillin by penicillinase, colour reactions, and volatility of aspergillic acid in steam. For final confirmation, isolation of the antibiotic may be necessary.

Several hundreds of moulds have been reported (Wilkins & Harris, 1942, 1943, 1944*a*, 1944*b*, 1944*c*; Furtado, 1944; Wiedling, 1944; Robbins, Hervey, Davidson, Ma & Robbins, 1945; Wilkins, 1946; and others) to produce diffusible substances inhibiting *Staph. aureus*, *Bact. coli*, or other bacteria. In the Aspergilli and Penicillia it appears that the action is in many cases due to one or more of a few well-known antibiotics which it would be desirable to be able to recognize early in the investigation of the culture fluid of any new organism. There are three main ways in which this can be done:

(1) by comparing the antibacterial spectrum of the substance with that of known antibiotics;

(2) by the method proposed by Stansly (1946) in which the unknown material is tested against one or more organisms sensitive to each known antibiotic, and also against derived strains of the same organism(s) in which resistance to the antibiotic has been induced;

(3) by comparison of the chemical and bacteriological behaviour of the unknown with that of known antibiotics.

The first method, besides requiring several standard organisms, is not very specific and is not applicable to mixtures of antibiotics. With the second method, the demonstration of non-identity is unambiguous, but any result suggesting identity must for the present be accepted with some reservations, since insufficient is known at present about cross resistance. Thus Eisman, Marsh & Mayer (1946) have shown that a staphylococcus made resistant to one of the three penicillins F, G or X acquired resistance of the same order of magnitude to the others. We had observed the same result independently. Admittedly the ability to distinguish between different penicillins is a very severe test of any method; but the method also suffers from the defect that a large number of standard cultures are required, at least two for each antibiotic which is to be tested for.

The third method has been used by many workers. The scheme given below is merely one of many possible and though it is certainly not claimed to be the best, it may serve as a starting point for workers entering the field.

The information on which the scheme is based is partly drawn from the literature. Where the necessary information was lacking or was presented in an unsuitable form, it was obtained by direct experiment.

Experimental

In interpreting the results, it must always be borne in mind that more than one antibiotic may be produced in a culture, either simultaneously or successively. For this reason the time of harvesting of the culture fluid may be important.

As a preliminary it is perhaps worth ruling out the possibility that the inhibition is due to hydrogen peroxide, by incubating the medium with a trace of blood or of a catalase preparation. Destruction of the activity indicates hydrogen peroxide.

The following tests are then carried out:

(1) The stability of the inhibitor is determined at 100° for 15 min., and at 20° for 30 min., at pH 2·0 and pH 9·5. The samples must, of course, be neutralized before assay, and any changes in volume taken into account.
(col. 1 of the Table).

(2) Samples of the culture fluid are shaken with equal volumes of ether at pH 2·0, 6·0–7·0 and 9·0. After separation, the aqueous phase is brought to pH 5·0–7·0 as quickly as possible, and a portion of the ether extract shaken with an equal volume of M/15-phosphate buffer of pH 6·8. The relative activity of the aqueous phase and the buffer extract of the ether is measured. Apparent loss of activity may be due to destruction at an unfavourable pH or the partition coefficient of the antibiotic being in favour of the ether at all pH values (col. 2 of the Table).

(3) Antibacterial specificity is examined. Only two strains of test bacterium have been used, namely, *Staphylococcus aureus* no. 6571 and *Bacterium coli* no. 86 of the National Collection of Type Cultures. The results recorded in column 6 of the Table were obtained with the cylinder-plate method (Heatley, 1944), the plates being surface-seeded with a 1:1000 dilution of a 16–24 hr. broth culture of the test organism.

In the intermediate class (nos. 8 and 9 in the Table) there may or may not be some degree of inhibition of *Bact. coli* as well as of *Staph. aureus*. The titres in col. 6 indicate merely the order of magnitude of the antibacterial effect.

From these screening tests it should be possible, with the aid of the Table, to conclude that the unknown inhibitor is not identical with any of the fourteen listed, that it is one or other of those in the list or that more than one active substance is present.

(4) In those cases where the screening tests narrow the possible identity to two or more of the substances given, certain simple chemical tests (column 3 of the Table) will distinguish between the possibilities. These tests, referred to by letter, are as follows:

		1				2			3	4	5	6	
		Stability*				Extracted into ether at pH			Con-firmatory tests†	Optical rotation	Melting-point (°)	Cylinder-plate assay. Dilution giving zone 20 mm. diameter‡	
		At pH 2·0		At pH 9·5									
Ref.	Antibiotic	20°	100°	20°	100°	2·0	6·0–7·0	9·0				*Staph. aureus*	*Bact. coli*
(1)	Penicillin	±	−	±	−	++	−	−	*a*	+	−	1 : 10^6	.
(2)	Helvolic acid	+	+	+	−	++	−	−	.	−	212	1 : 160,000	<1 : 1000
(3)	Mycophenolic acid	+	+	+	+	++	−	−	*c*	0	141	1 : 32,000	<1 : 200
(4)	Proactinomycin	+	±	−	−	−	−	++	.	+	.	1 : 5000	<1 : 100
(5)	Citrinin	+	±	+	±	++	−	−	*c, e*	−	166–170	1 : 16,000	<1 : 500
(6)	Gliotoxin	+	+	−	−	++	++	−	.	−	221	1 : 32,000	<1 : 500
(7)	Puberulic acid	+	+	+	+	++	−	−	*c*	0	316–318	1 : 5000	<1 : 500
(8)	Fumigatin	+	+	+	.	++	−	−	*b*	0	116	1 : 1000	<1 : 1000
(9)	Spinulosin	+	+	+	±	++	−	−	*b*	0	200–201	1 : 250	<1 : 250
(10)	Anhydro-3-hydroxymethylene-tetrahydro-pyrone-2-carboxylic acid§	+	+	±	−	+	+	−	.	0	111	1 : 10,000	1 : 10,000
(11)	Aspergillic acid	+	+	+	+	++	−	−	*f*	+	96	1 : 1000	1 : 1000
(12)	Penicillic acid	+	+	+	+	++	−	−	*d*	0	65	1 : 1000	1 : 1000
(13)	Kojic acid	+	+	+	+	++	−	−	*c*	0	150	1 : 150	1 : 150
(14)	Streptomycin	+	+	+	−	−	−	−	.	0	.	1 : 2000	1 : 2000

* In this column + indicates little or no destruction; ± indicates some destruction; − indicates almost complete destruction.

† See § 4, p. 235.

‡ Plates surface-seeded with 1 in 1000 dilution of 16–24 hr. broth culture of *Staph. aureus* N.C.T.C. no. 6571, or *Bact. coli* N.C.T.C. no. 86.

§ Variously named expansine, clavatin, clavacin, claviformin, patulin, penicidin.

References to properties and isolation procedure

(1) Abraham & Chain (1942).
(2) Chain *et al.* (1943).
(3) Clutterbuck & Raistrick (1933); Florey *et al.* (1946).
(4) Gardner & Chain (1942).
(5) Heatherington & Raistrick (1931); Tauber *et al.* (1942).
(6) Weindling (1941); Johnson *et al.* (1943).
(7) Oxford *et al.* (1942*b*).
(8) Anslow & Raistrick (1938*a*); Oxford & Raistrick (1942).
(9) Birkinshaw & Raistrick (1931); Anslow & Raistrick (1938*b*).
(10) Chain *et al.* (1942).
(11) White & Hill (1943); Menzel *et al.* (1943).
(12) Birkinshaw *et al.* (1936); Oxford *et al.* (1942*a*).
(13) Birkinshaw *et al.* (1931); Jennings & Williams (1945).
(14) Schatz *et al.* (1944); Schenck & Spielman (1945).

(*a*) The activity of the culture medium is measured after incubation in the presence and absence of penicillinase (e.g. Duthie, 1944). Destruction of the activity indicates penicillin.

(*b*) Fumigatin and spinulosin in the concentrations which can be detected by the cylinder-plate method give deep purple solutions. In mould cultures, however, the substances may by reduction give a paler brownish colour; on shaking in air the purple colour is restored. The purple colour is also reversibly discharged in acid solution.

(*c*) A drop of dilute ferric chloride solution is added to the culture medium at neutral or faintly acid pH; *a red colour* indicates kojic acid; *a red-brown colour* indicates puberulic or puberulonic acids; *a blue-violet colour* indicates mycophenolic acid; *a brown precipitate*, soluble in excess of $FeCl_3$ solution, indicates citrinin.

(*d*) 2–3 volumes of strong ammonia are added. A red colour suggests penicillic acid.

(*e*) On acidification, a bright yellow colour which may or may not be accompanied by a yellow precipitate, indicates citrinin.

(*f*) Aspergillic acid is volatile in steam.

The next stage is to confirm the tentative identification. Some tests (e.g. the penicillinase test for penicillin) have a high degree of specificity, but in other cases it may be necessary to isolate the inhibitor and apply the usual chemical criteria of identity—mixed melting-point, etc. The colour tests are suggestive, not specific; taken in conjunction with other tests they may be helpful. The presence of pigment in the medium or a low concentration of the antibiotic may make the tests of value only after some purification.

The papers cited in the Table provide further tests or isolation procedures for the antibiotics listed.

One of us (F. J. P.) is indebted to the Agricultural Research Council for a personal grant. Our thanks are due to Professor H. Raistrick for samples of spinulosin and puberulic acid, and to Dr P. W. Brian for a sample of gliotoxin; and to Miss Jean Stewart for technical assistance.

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A Selective Medium for Pleuropneumonia-like Organisms

By D. G. ff. EDWARD

The Wellcome Veterinary Research Station, Frant, Sussex

SUMMARY: The action of several bacteriostatic substances on strains of pleuropneumonia-like organisms has been tested. Suitable concentrations of thallium acetate and penicillin in the media were inhibitory for ordinary bacteria, but allowed the pleuropneumonia-like organisms to grow in pure culture and facilitated their primary isolation. These two bacteriostatic substances were also useful for the isolation of strains on plate cultures. The addition of thallium acetate alone to media used for maintaining strains in subculture diminished the number of contaminations.

The media gave variable results, depending on the batch of serum used for enrichment. The routine addition of fresh yeast extract yielded consistently good growths of all strains of pleuropneumonia-like organisms tested. The effective factor in the yeast was not the 'V' factor required by Pfeiffer's influenza bacillus.

Organisms morphologically and culturally similar to the pathogenic agent of contagious bovine pleuropneumonia have been isolated recently from a number of widely different sources, including the human genital tract (Dienes, 1940; Beveridge, 1943). Failure to recognize this group until lately has been largely due to the peculiar properties of the organisms, especially their exacting cultural requirements, the slowness and paucity of their growth, and the peculiarities of their morphology. Surface colonies are very small and slow to appear and are thus easily overgrown by other organisms. Moreover, certain pleuropneumonia-like organisms associated with infectious catarrh of the upper respiratory tract in mice do not give surface colonies on primary isolation, and can only be isolated by using fluid or semi-solid media (Edward, 1940, 1947). A medium has therefore been sought which would inhibit ordinary bacteria while allowing pleuropneumonia-like organisms to grow in pure culture.

Beveridge (1943) found that pleuropneumonia-like organisms from the human genital tract were relatively resistant to sulphanilamide and used media containing this substance for primary isolation; he also noted growth in media containing gentian violet. The experiments of Powell & Rice (1944), in which penicillin failed to protect mice against arthritis caused by a pleuropneumonia-like organism, suggested that this group might be resistant to penicillin.

Cultural methods

Basal liquid media were prepared from ox heart infusion broth with 1% added peptone, adjusted to pH 8·0. The basal medium gradually deteriorated on storage and yielded good growths only if used during periods of less than about three months. The final medium was made by adding 20% (v/v) horse serum and 10% (v/v) of a yeast extract (≡250 g./l.), with sufficient sterile caustic soda to bring the final pH to 8·0. To get rid of precipitable phosphates, broth was prepared in bulk at pH 8·4, incubated overnight at 37° and then Seitz-filtered through a clarifying pad. Finally it was adjusted to pH 8·0 and tubed. The yeast extract was prepared by adding 50 g. of brewer's yeast to 200 ml. distilled water and boiling until frothing ceased; and was sterilized by Seitz filtration. It was used fresh, but was still suitable after storage in the refrigerator for one week.

Three types of media were used, broth, agar plates and sloppy agar. For solid media 2% agar (or 1% New Zealand agar) was added. Sloppy agar (0·3% agar), introduced by Beveridge (1943), was found particularly useful for the isolation and maintenance of strains. In it the growth of pleuropneumonia-like orgainisms varied from a fine granularity, when the inoculum was heavy, to more definite macroscopic colonies, as described by Beveridge. As a routine, stock cultures were maintained in duplicate both in sloppy agars and on plates. For primary isolation of strains capable of giving macroscopic surface colonies, plates had the advantage because colonial appearances of all members of the pleuropneumonia-like group of organisms were so typical that they allowed more rapid and reliable identification.

The L5 organism (Findlay, Klieneberger, MacCallum & Mackenzie, 1938) and the strains from mouse catarrh were shown to be obligatory aerobes. In sloppy agar they grow only in the upper part of the medium. Growth occurred in an atmosphere of 10% carbon dioxide in air, but was poor. Strains from the human genital tract grew equally well aerobically, anaerobically and in 10% carbon dioxide in air.

The 'Yeast Factor'. The use of yeast extract followed the observation that media containing only broth, agar, peptone and horse serum gave variable results depending on the batch of horse serum; sometimes growth was scanty or almost completely absent. Where growth was poor, colonies were larger and more numerous around contaminating colonies of staphylococci. Growth was improved by the addition of freshly prepared filtrates of cultures of these staphylococci. Yeast extract produced a similar enhancement of growth for the L5 organism, strains from mouse catarrh and strains from the human genital tract, but not for the saprophytic sewage organisms of Laidlaw & Elford (1936). Subsequently yeast extract was added to media as a routine and consistently good growths were obtained.

The factor in the yeast extract necessary for growth withstood heating in the autoclave at 120° for 60 min.; it was not destroyed by boiling either at pH 4·2 or at pH 8·5. Thus the factor was not the 'V' factor, coenzymes I or II of Lwoff & Lwoff (1937). It could not be removed from the extract, nor from a sample of horse serum in which it was abundant, by adsorption on charcoal or by dialysis. Other additions to media used for growing pleuropneumonia-like organisms have been recommended. Klieneberger (1936) used boiled blood and Beveridge (1943) extracts of liver. It appeared that neither of these contained a factor similar to that in yeast, because, when a batch of horse serum was used which needed the addition of yeast extract for growth to occur, it was not possible to obtain growth by replacing the yeast by either of them.

Action of bacteriostatics

Several commonly used bacteriostatic substances were tested for their inhibitory action on the L5 organism and on a pleuropneumonia-like organism isolated from mice with catarrh, and also on certain representative bacteria, namely *Staph. albus*, *Staph. aureus*, *Strep. faecalis*, *B. subtilis*, *Bact. coli*, *Proteus vulgaris* and an avirulent diphtheroid organism. The tests were carried out in 10 ml. amounts of sloppy agar. Inocula were 0·1 ml. of cultures of pleuropneumonia-like organisms in broth or sloppy agar, and one loopful of 1:1000 dilutions of overnight cultures of the bacteria.

Sodium azide at a concentration of 1:2000 inhibited the L5 organism but was not inhibitory at 1:5000; the latter concentration did not prevent growth of any of the representative bacteria (Table 1). Potassium tellurite was markedly inhibitory for L5, only feeble growth occurring in a concentration of 1:50,000. L5 was completely inhibited by 1:100,000 brilliant green and by 1:500,000 gentian violet; there was feeble growth in 1:1,000,000 gentian violet. Neither strain of pleuropneumonia-like organism was inhibited by thallium acetate at concentrations of 1:1000 or less, nor by concentrations of penicillin as high as 390 Oxford units/ml.

Although these tests showed that a medium containing 60 units of penicillin /ml. prevented growth of all the representative bacteria tested, it seemed likely that strains of aerobic spore-bearing bacilli and non-sporing Gram-negative bacilli with greater resistance to penicillin, or actually able to inactivate it, would sometimes be encountered in practice. Accordingly a selective medium was made containing both penicillin and thallium acetate, the latter being highly bacteriostatic for aerobic spore-bearers and Gram-negative bacilli. The lowest concentration of thallium acetate found to inhibit *P. vulgaris* was 1:2000. For *Strep. faecalis* 60 units/ml. of penicillin was inhibitory, but not 12 units/ml. The concentrations chosen for use were therefore 1:2000 thallium acetate and about 60 units/ml. of penicillin. Recently Beveridge, Campbell & Lind (1946) described the isolation in pure culture of pleuropneumonia-like organisms from the human genital tract in a semi-solid medium containing 20 units of penicillin/ml.

The value of thallium acetate was shown in the present investigation by inoculating sloppy agars containing 98 units of penicillin/ml. and 1:2000 thallium acetate, in parallel with sloppy agars containing penicillin alone, using as inocula material heavily contaminated with sporing and other bacilli, such as dust, bovine faeces, uterine discharges, etc. Bacteria grew out in the media containing penicillin only, but not in those containing penicillin and thallium acetate.

Thallium acetate was made up as a 10% solution, sterilized by autoclaving and added to the final medium before or after tubing. It was not found necessary to diminish the concentration of sodium chloride in the medium, as suggested by McKenzie (1941). Any precipitate which formed at first subsequently redissolved. In serum agar plates, 1:2000 thallium acetate caused some degree of inhibition of the pleuropneumonia-like organisms and for plates a concentration of 1:8000 was subsequently used. It is not so essential to have plates completely inhibitory to the ordinary bacteria. Solutions of penicillin (one 10,000 unit tablet dissolved in 1 ml. of sterile distilled water and 0·1 ml. of the solution added to 10 ml. of medium) were freshly prepared and added immediately before inoculation. Penicillin was applied to plates by spreading two drops of a solution containing approximately 1000 units/ml. across half the plate, allowing to dry and using immediately.

Table 1. *Action of bacteriostatics on strains of pleuropneumonia-like organisms and on certain representative bacteria*

Final concentration of bacteriostatic substance in the medium	Pleuropneumonia-like organisms								
	L 5	Strain from mouse catarrh	*Staph. albus*	*Staph. aureus*	*Strep. faecalis*	A diphtheroid organism	*B. subtilis*	*Bact. coli*	*Proteus vulgaris*
Sodium azide:									
1 : 2000	−	.	.	.	.	.	.	.	.
1 : 5000	+	+	+	+	+	+	+	+	+
1 : 10,000	+	+	+	+	+	+	+	+	.
Potassium tellurite:									
1 : 20,000	−	.	.	.	.	.	.	.	.
1 : 50,000	(+)	.	.	.	.	.	.	.	.
Thallium acetate:									
1 : 500	−	−	.	.	.	.	.	.	.
1 : 1000	+	+	+	+	+	+	−	−	−
1 : 2000	+	+	+	+	+	+	−	−	−
1 : 4000	.	.	.	.	.	.	−	−	+
1 : 8000	.	.	.	.	.	.	+	+	+
Gentian violet:									
1 : 500,000	−	(+)	−	−	+	−	−	+	.
1 : 1,000,000	(+)	+	.	.	.	.	.	.	.
Brilliant green:									
1 : 100,000	−	.	.	.	.	.	.	.	.
Penicillin:									
390 units/ml.	+	.	.	.	.	.	.	.	.
98 units/ml.	+	+	−	−	−	.	−	−	−
60 units/ml.	+	+	.	.	−	.	.	.	+
12 units/ml.	+	+	−	−	+	−	−	+	+

+ Represents good growth, equal to that in the control.
(+) Represents poor growth, less than that in the control.
− Represents complete inhibition of growth.
. Indicates that no observation was made.

Results

In six preliminary tests sloppy agars containing thallium acetate and penicillin were inoculated both with swabs taken from the human throat and with stock cultures of pleuropneumonia-like organisms. In all cases bacteria failed to grow and the pleuropneumonia-like organisms were recovered in pure culture. A series of tenfold dilutions of a culture of L5 seeded into bottles of this medium grew as well as in sloppy agars without penicillin or thallium acetate.

Sloppy agars and broths containing thallium acetate and penicillin have been used in approximately 100 attempted isolations of pleuropneumonia-like organisms from the noses of mice and from the human and bovine genital tract, using as inocula material obviously contaminated with bacteria. Only four times have ordinary bacteria grown; on two of these occasions the bacterial load in the inoculum (ox semen) was particularly heavy, and on another occasion the broth only, and not the sloppy agar, grew bacteria. There have been numerous isolations of pleuropneumonia-like organisms in the media from the noses of mice (Edward, 1947) and from the human and bovine genital tract. Unfortunately it was seldom possible to obtain material infected with pleuropneumonia-like organisms but not contaminated with bacteria, in order to compare media with and without bacteriostatics. However, the few observations made did not suggest that the selective media inhibited any of the naturally occurring strains.

Swabs from the human and bovine genital tract were also plated in duplicate on media with and without 1:8000 thallium acetate; half of each plate was spread with penicillin. Several isolations of pleuropneumonia-like organisms were made and there was no evidence of inhibition by penicillin or thallium acetate.

Sloppy agars and plates containing 1:2000 and 1:8000 thallium acetate respectively, without the addition of penicillin, proved useful for the maintenance and examination of stock cultures. Thallium acetate had the advantage over penicillin that it could be added when the media was made and, although it did not inhibit all bacteria, it was bacteriostatic for some of the most troublesome contaminants, such as the aerobic spore-bearing bacilli and Gram-negative bacilli.

I am indebted to Dr E. Klieneberger-Nobel and Dr W. J. Elford for supplying me with strains of pleuropneumonia-like organisms.

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Some Observations on the use of Penicillinase in Sterility Tests for Penicillin

By M. PILE, D. D. STEWART and A. F. B. STANDFAST

The Bacteriological Laboratory, The British Drug Houses Ltd., London

SUMMARY: Penicillinase preparations strong enough to destroy all the penicillin in 1 hr. or less at room temperature should be used in carrying out sterility tests on solid penicillin. Otherwise, penicillin-sensitive contaminants may be killed by the penicillin before it is destroyed and samples containing them will be sterile by the test. A convenient unit of penicillinase is the quantity which will destroy 100 units of penicillin in 1 ml. in 1 hr. at room temperature (24°).

The usual sterility tests when applied to penicillin can show the presence only of penicillin-resistant organisms, any penicillin-sensitive organisms present in the dry penicillin powder being killed when the penicillin is dissolved. For obvious reasons it is not possible to dilute the penicillin to a concentration at which it is no longer active, for the quantities of broth necessary would be unmanageable.

In order to overcome this difficulty, Harper (1943) suggested the use of penicillinase in sterility tests, and Proom (1945) described the preparation of penicillinase from a paracolon bacillus in a strength suitable for sterility tests of high-potency penicillin. Lawrence (1943) reported that 'clarase' was a satisfactory agent for destroying penicillin in sterility tests, though later work has shown that this was probably due to a penicillinase in the 'clarase'.

The usefulness of penicillinase in sterility tests depends on its ability to destroy the penicillin before the penicillin has killed *any* of the penicillin-sensitive organisms which might be present. Although in the literature there are references to the fact that 'small numbers' of organisms were not affected by the penicillin in penicillin-penicillinase mixtures, we were not satisfied with the evidence, particularly as most workers use undetermined numbers of the test organisms to test the inactivation of the penicillin, assuming, if on incubation the test organism grew out, that the penicillin had been destroyed.

Some of our preliminary experiments indicated that though such inocula might multiply in these mixtures of penicillin and inactivating agent, some of the bacteria might have been killed before the penicillin was destroyed.

The experiments described below were designed to demonstrate the degree to which this killing occurred in the conditions of routine sterility tests. They show that, in order to destroy all penicillin activity before any contaminants are killed, very large quantities of penicillinase are necessary.

Material and methods

Penicillin. Samples of commercial penicillin calcium salt (about 500 units/mg.) were used. Penicillin was assayed by the routine plate method used in this laboratory.

Penicillinase. Batches of penicillinase were prepared from a paracolon bacillus by the method of Proom (1945) and from *B. subtilis* by the methods of Coulthard (personal communication), Smith (1945) and those devised in this laboratory.

Viable counts. All viable counts were made in roll tubes containing 1 ml. of double strength agar, to accommodate the inoculum of 1 ml. per roll tube necessary to demonstrate small numbers of organisms. Six replicates of each count were made.

All roll tubes were incubated at 37° and counted after 1 day, and again after 7–14 days, in case the penicillin had produced an abnormally long lag phase in some of the bacteria. In no experiment, however, was this the case. For example, three tubes having 39, 42 and 50 colonies after 1 day were counted as 39, 39 and 49 after 10 days' incubation.

The assay of penicillin-penicillinase mixtures

Residual penicillin in penicillin-penicillinase mixtures can be titrated by the cup-plate method. The continued action of the penicillinase during the incubation of the test plate is offset by the fact that penicillin diffuses through the agar at a much greater rate than penicillinase. For example, when lots of 100 units of penicillin were mixed with 1, 2, 3 and 5 arbitrary units (see p. 250) of penicillinase, the mixture immediately diluted 1 in 100 and placed in the cups, the differences between the mean diameters of the inhibition rings (average of six replicates) produced by the control penicillin and the various penicillin-penicillinase mixtures were within the experimental error of the plate assay. The penicillin, having diffused away from the penicillinase through the agar, was not destroyed during incubation of the cup-plate.

This method of demonstrating penicillin activity has given us more consistent results than did the titration of penicillin after extraction from the mixtures. Five hundred units of penicillin in 5 ml. were plunged into ice-cold water, adjusted to pH 3·0 and shaken for 15 min. with an equal volume of chloroform. The chloroform after separation was shaken with an equal volume of sodium bicarbonate solution. The process was repeated on a sample of the penicillin with 500 units/ml. of penicillinase. All the samples were diluted so as to contain approximately 1 unit/ml. and assayed by the plate method. The ring diameters were:

Original penicillin	21·5 mm. ≡ 100 units/ml.
Extracted penicillin	18·6 mm. ≡ 86 units/ml.
Extracted penicillin-penicillinase	17·0 mm. ≡ 79 units/ml.

It is probable that the 86 – 79 = 7 units lost in the penicillinase mixture were destroyed while the solutions were being adjusted to pH 3·0 and perhaps during the shaking with chloroform. All similar experiments showed this loss.

When penicillin is incorporated in the agar in a test plate and penicillinase put in the cups, rings of staphylococcal growth are obtained where the penicillinase has diffused into the agar. For plates containing 1 unit of penicillin/ml. of agar, solutions containing 2000 arbitrary units of penicillinase/ml. are needed to get rings of a diameter (about 18 mm.) comparable to that given by 1 unit of penicillin (about 21 mm. diam.) in the ordinary cup-plate titration.

Table 1. *The killing of staphylococci in penicillin-penicillinase mixtures at room temperature*

Composition of mixtures		Samples taken at (hr.)					
Penicillin total units	Penicillinase (ml.)	0	1	2	4	6	24
		Mean viable counts and residual penicillin/ml.					
0	0	78	68	70	57	55	∞
5400	0	78	0	0	0	0	0
Residual penicillin		.	+	+	+	+	+
0	4	78	74	72	69	74	∞
5400	4	78	14	17	30	37	∞
Residual penicillin		.	+	+	±	0	0
0	2	78	78	63	81	74	∞
5400	2	78	2	1	7	8	24
Residual penicillin		.	+	+	+	0	0
0	1	78	72	71	65	74	∞
5400	1	78	0	0	0	0	0
Residual penicillin		.	+	+	+	+	0
0	0·5	78	74	74	62	68	∞
5400	0·5	78	0	0	0	0	3
Residual penicillin		.	+	+	+	+	0

∞ = more than 1 million organisms/ml.
Penicillin assay. + = more than 0·5 unit/ml.
± = about 0·1 unit/ml.
0 = no demonstrable penicillin.

Solutions of penicillinase of such strength that 1 ml. will destroy 100 units of penicillin do not give measurable rings on agar plates containing 1 unit of penicillin/ml.

It is therefore possible to titrate the residual penicillin in mixtures by the plate method.

Rate of destruction of penicillin by penicillinase

At room temperature. In the first series of experiments, a penicillinase was used of such a strength that 0·5 ml. would destroy 6000 units of penicillin in 24 hr. Quantities from 0·5 ml. to 4·0 ml. were added to 5400 units of penicillin in broth. *Staph. aureus* (Oxford, Heatley strain) was added to give a final concentration of 80 viable cocci/ml. The final volume of broth in each tube was 66 ml. and therefore the final concentration of penicillin was 82 units/ml. The tubes were stood at room temperature and at the times stated sampled without further dilution for viable counts and penicillin assays. The results of a typical experiment are shown in Table 1.

It will be seen that even with a large concentration of penicillinase, the penicillin is not destroyed quickly enough to allow all the organisms to survive. The tube containing 4 ml. of penicillinase shows a drop from about 78 to 37 cocci/ml. in 6 hr., by which time there was no penicillin left to be carried over into the roll tubes; in the tube with 2 ml. of penicillinase there were only 8 cocci left after the destruction of the penicillin by the penicillinase. It should be noted that penicillinase alone showed no bacterial or bacteriostatic activity in the concentrations used.

Table 2. *The killing of staphylococci in penicillin-penicillinase mixtures at 37°*

The figures show increased rates of reaction in penicillin-penicillinase mixtures at 37° as compared with room temperature (see Table 1).

Composition of mixtures		Samples taken at (hr.)					
Penicillin total units	Penicillinase (ml.)	0	1	2	4	6	24
		Mean viable count and residual penicillin/ml.					
0	0	48	46	40	300	∞	∞
5400	4	48	31	36	33	511	∞
Residual penicillin		·	+	±	0	0	0
5400	2	48	4	8	29	129	∞
Residual penicillin		·	+	+	0	0	0
5400	1	48	0	0	0	2	∞
Residual penicillin		·	+	+	±	0	0
5400	0·5	48	0	0	0	0	∞
Residual penicillin		·	+	+	+	+	0
5400	0	48	0	0	0	0	0
Residual penicillin		·	+	+	+	+	+

Symbols as in Table 1.

At 37°. Three experiments, one of which is set out in detail in Table 2, were carried out at 37° to see if the penicillin-penicillinase reaction would be accelerated and the exposure of the cocci to the penicillin thereby shortened.

With 4 ml. of penicillinase, which destroyed all the penicillin in less than 4 hr., the viable count had fallen from about 48 cocci/ml. at 0 hr. to 33 cocci/ml., compared with the control tube at 4 hr. with 300 cocci/ml. With 1 ml. of penicillinase, which destroyed all the penicillin in 6 hr., the viable count had been reduced to 2 cocci/ml. though in 24 hr. these had grown out to more than 1 million. This experiment illustrates a fallacy of penicillin-penicillinase experiments in which viable counts are not made, for at 24 hr. the bacterial density in this tube was equal to that in the control, although a large number of cocci had been killed by the penicillin before it was destroyed by the penicillinase.

Experiments with staphylococci, streptococci and B. subtilis

The experiments showing that staphylococci may be killed by penicillin in the presence of large quantities of penicillinase were repeated with other organisms (Table 3).

Table 3. *The killing of staphylococci, streptococci and* B. subtilis *in penicillin-penicillinase mixtures at room temperature*

Organism	Composition of mixtures		Samples taken at (hr.)				
	Penicillin total units	Penicillinase (ml.)	0	1	2	4	6
			Mean viable count and residual penicillin/ml.				
Staphylococcus aureus (strain Heatley)	0	1	20	20	22	20	24
	4200	1	20	12	18	15	12
	Residual penicillin		.	+	+	0	0
Streptococcus pyogenes (strain F, Richards)	0	1	40	43	38	40	42
	4200	1	40	0	0	0	0
	Residual penicillin		.	+	+	0	0
B. subtilis (strain B.D.H.)	0	1	16	16	15	17	19
	4200	1	16	1	8	4	8
	Residual penicillin		.	+	+	0	0

Symbols as in Table 1.

Here again, the destruction of penicillin by penicillinase was not sufficiently rapid (Table 3). In 4 hr., by which time all the penicillin had been destroyed, the staphylococci had been diminished from 20 to 15, the streptococci had been killed and the *B. subtilis* counts reduced from 16 to 4 organisms/ml. The last figure is too low to be counted accurately by the roll tube method, but the regularity of the count, 2, 3, 3, 3, 5, 7, average 4 colonies/roll tube, gives an indication of the behaviour of *B. subtilis*.

The use of large quantities of penicillinase in sterility tests

From the foregoing experiments it is clear that penicillinase in much larger quantities than has been customary must be used for a satisfactory sterility test. A series of tests were set up with penicillinase of a strength such that 2 ml. would destroy 6000 units of penicillin in 1 hr. at room temperature. Residual penicillin was accurately titrated from 50 units/ml. downwards. These tests gave similar results, and one test is set out in full (Table 4).

With 2 and 4 ml. of penicillinase, which destroyed all the penicillin in 1 hr., there was no significant killing of staphylococci, but with 1 ml., which decreased the penicillin from 92 units/ml. to 0·8 unit/ml. in 2 hr. and destroyed it all in 4 hr., the viable count was decreased to 25 cocci/ml. in 4 hr. With 0·5 ml. of penicillinase which destroyed all the penicillin in 6 hr., the viable count was only 8.

Repeated tests with penicillinase at this strength, i.e. 4 or 8 ml. of a strength such that 2 ml. would destroy all the penicillin present in 1 hr. or less, showed that this is a satisfactory strength to ensure that none of the organisms present is killed by the penicillin.

DISCUSSION

These experiments show that if penicillinase is employed it must be used in a concentration sufficient to destroy the penicillin before it has killed any contaminating organisms, if falsely negative results are to be avoided. The importance of this fact cannot be stressed too highly when very small numbers of penicillin-sensitive contaminating organisms are to be detected.

Table 4. *The rapid inactivation of penicillin by large quantities of a suitable penicillinase at room temperature*

Test organism: *Staphylococcus aureus.*

Composition of mixtures		Samples taken at (hr.)					
		0	1	2	4	6	24
Penicillin total units	Penicillinase (ml.)	Mean viable count and residual penicillin/ml.					
0	0	31	32	33	35	49	∞
5400	0	31	0	0	0	0	0
Residual penicillin		.	++	++	++	++	++
0	4	31	33	27	36	47	∞
5400	4	31	33	33	38	39	∞
Residual penicillin		.	0	0	0	0	0
0	2	31	31	33	40	51	∞
5400	2	31	29	29	35	50	∞
Residual penicillin		.	0	0	0	0	0
0	1	31	27	26	33	49	∞
5400	1	31	8	12	25	26	∞
Residual penicillin		.	12	0·8	0	0	0
0	0·5	31	33	32	35	67	∞
5400	0·5	31	1	1	2	8	34
Residual penicillin		.	29	14	0·8	0	0

Penicillin assay. ++ = more than 50 units/ml. Other symbols as in Table 1.

It is customary to assay penicillinase in terms of the quantity of the preparation under test which will destroy 100 units of penicillin. However, the time taken for the destruction and the temperature are important, and the potency of the penicillinase should be reported as the quantity which will destroy 100 units of penicillin in 1 ml. *in a given period and at a given temperature.* From the practical viewpoint of sterility testing, we suggest that the time of the reaction should not exceed 1 hr. and that room temperature is better than 37°. At 37°, though the destruction of the penicillin is more rapid and the amount required rather less, the action of penicillin on bacteria is also more rapid and there is a greater danger of contaminants being killed.

A potent and rapidly acting penicillinase is necessary for satisfactory sterility tests, and we suggest that a convenient unit of penicillinase activity is the quantity of a preparation that will destroy 100 units of penicillin in 1 ml. in 1 hr. at room temperature. A penicillinase like that used in the experiments described in Table 4, if used in excess, would probably protect the most penicillin-sensitive contaminant.

We wish to thank the Directors of the British Drug Houses Ltd. for permission to publish this paper.

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The Preservation of Bacteria by Drying

By LORD STAMP

The Microbiological Research Department, Porton, and
the British Postgraduate Medical School, London

SUMMARY: A quantitative study of the survival of vegetative bacteria on drying in various suspending media led to a simple method for preserving bacterial cultures. Bacterial cells are suspended in melted nutrient gelatin containing ascorbic acid or sodium ascorbate in concentration of 0·25–0·5 %. Small quantities are dried over P_2O_5 at pressures of 100–300 mm. of mercury and stored *in vacuo* over P_2O_5 at room temperature. A wide range of bacterial species of medical and veterinary importance was preserved by this method for 4 years. The slow decline in the number of viable organisms and the high percentage survival rate at 4 years indicate the likelihood of survival for a much longer period.

The virulence of a number of pathogenic species was successfully maintained in the dry preparation. The method might well be applied to the preservation of living vaccines.

The survival rates with *Chromobacterium prodigiosum* dried by this method were better than those in preparations subjected to rapid freeze-drying processes. They were, however, unsatisfactory with a few species such as *Vibrio cholerae* and *Neisseria meningitidis.*

A number of methods have been described for the preservation of stock cultures of bacteria by drying. The simplest consists in drying the organisms suspended in culture fluid or resuspended in saline, serum or blood in a desiccator *in vacuo* over dehydrating agents such as H_2SO_4 or P_2O_5. The suspensions are dried on sterile coverslips, filter paper, in small test-tubes, or more conveniently in sterile ampoules which can subsequently be sealed off *in vacuo.* Successful results with a variety of bacterial species have been reported by Heim (1905, 1907, 1922), Brown (1925, 1932), Harris & Lange (1933), Otten (1930, 1932), Pauli (1932), and Mackie & McCartney (1945). Some pathogens have also been preserved satisfactorily by similar drying of the spleens taken from infected animals. Others recommend freezing the bacterial suspension immediately before and during the drying process by immersing the container in an ice and salt mixture, or in a mixture of CO_2 snow and glycerol placed in the desiccator (Shackell, 1909; Rogers, 1914; Swift, 1921, 1937; Morton & Pulaski, 1938). A more elaborate freeze-drying process was developed by Elser, Thomas & Steffen (1935) and Flosdorf & Mudd (1935, 1938) for the preservation of biological products, including micro-organisms. In this so-called 'lyophile' process the bacterial suspension is delivered into ampoules and frozen by immersion in salt and ice mixture or CO_2 snow. The ampoules are then attached to a manifold and the moisture is drawn off by means of an efficient vacuum pump, such as the Cenco-Megavac or Hyvac, and trapped by condensation in a vessel immersed in CO_2 snow or by means of a chemical desiccant chamber containing anhydrous calcium sulphate. Traps containing P_2O_5 are used also to collect the last traces of moisture carried over. Drying is complete in a very short period and the ampoules are then sealed off *in situ* under high vacuum. This method is said to give the most satisfactory results, particularly with delicate organisms.

However, in little of the work published on the drying of bacteria has any attempt been made to determine systematically the conditions most favourable for survival. This can be done satisfactorily only by quantitative methods in which the percentage of organisms surviving desiccation under different conditions is accurately estimated. In this paper a study has been made on these lines of some of the factors which might be expected to influence survival (such as the use of different suspending media) and of the two methods in common use for preserving stock cultures by drying, namely, simple drying in the unfrozen state in a desiccator over dehydrating agents *in vacuo*, and the more rapid freeze-drying 'lyophile' process. As a result a simple drying technique has been developed and used to preserve a large number of bacterial species.

METHODS

Preparation of the bacterial suspension. The organism under test was grown on a suitable agar medium in Petri dishes or Roux bottles. The latter proved preferable in view of the lesser likelihood of contamination. In one or two experiments (e.g. with *Pasteurella pestis*) the suspension was made from a broth culture. The growth was removed by scraping off with a sterile glass slide or by washing off and centrifuging. The packed cells were thoroughly emulsified in the suspending medium. A wide range of concentrations of bacterial cells was used, but in most cases a thick suspension containing 5×10^9 to $1{\cdot}5 \times 10^{10}$ organisms/ml. of suspending medium was used. With profusely growing organisms such as the Salmonellae, these concentrations were attained when the growth from three agar plates or one Roux bottle, grown for 24 hr. at 37° on tryptic meat agar or CCY agar (Gladstone & Fildes, 1940), was added to 2·5–10 ml. of suspending medium. In the majority of tests the suspending medium was nutrient gelatin, containing peptone (Evans, Sons, Lescher & Webb) 1·0%, NaCl 0·5%, Lemco beef extract 0·4%, and Coignet gold label gelatin 10·0%. The first three ingredients were made up double strength, the pH adjusted to 9·3 to precipitate phosphates which were removed and then readjusted to pH 7·6; an equal volume of a 20% solution of gelatin was then added. The medium was cleared with egg albumin, steamed for 30 min., shaken, steamed for a further 2 hr., filtered, the pH adjusted to 7·6 and the medium autoclaved at 115° for 15 min.

Other suspending media were: fresh defibrinated horse blood, fresh sterile rabbit serum heated at 60° for 1 hr., 2·5% solution of mucin in distilled water, 2·0% solution of polyvinyl alcohol in distilled water sterilized at 100°. The pH of each was checked and adjusted when necessary to *c.* 7·0 before use. Solutions of ascorbic acid were made up in the suspending medium under test or in distilled water in ten times the strength required and heated to 100° for a few seconds to sterilize. One part of concentrated solution was then added to nine parts of thick bacterial suspension and mixed thoroughly.

Drying the bacterial suspension

Drying in the unfrozen state in a desiccator over P_2O_5 *in a partial vacuum.* In early experiments the bacterial suspension was delivered into Petri dishes in 1 ml. quantities. But in the great majority of experiments it was delivered on to a sterile waxed surface as discrete drops by means of a 'standard' dropping pipette, calibrated to deliver 36 drops of water/ml. under controlled conditions. The waxed surface was made by dipping a filter paper into hot melted paraffin wax to sterilize, allowing to cool and placing in a Petri dish. In some tests the drops were delivered directly into one ml. vials with wide necks. The samples were placed in a desiccator containing P_2O_5 and dried at a pressure of 100–300 mm. Hg. Drying was continued with excess P_2O_5 for 2–3 days at room temperature, after which no further appreciable loss in weight was found to occur. Before opening, the desiccator was slowly filled with air filtered through sterile cotton-wool. On drying the drops formed thin disks of uniform size which were carefully scraped off the filter paper, transferred to a suitable sterile container and stored under a cotton-wool plug in a desiccator over P_2O_5, usually at low pressure. For subculture a disk was removed from the container by touching with a moistened platinum loop, to which it readily adhered, transferred to broth and warmed to redissolve. One loopful was then subcultured in fresh broth or on to a solid medium. There was little trouble with contamination of samples provided all possible aseptic precautions were taken during drying. Small numbers of contaminants present in the dried disk were presumably diluted out when it was redissolved and subcultured. The desiccators were stored at room temperature during the first 2-year period, namely, laboratory temperatures of 18–21°, except for brief periods during the summer months. During the second 2 years they were kept in a cool cellar at somewhat lower temperatures.

Drying by the lyophile process. The method of Flosdorf & Mudd (1935) was used.

Drying by the McFarlane process. The McFarlane (1942–3) process differs from other freeze-drying processes in that the material to be dried is held frozen to a metal surface. A coil of tubing was used, through which brine was circulated at −40°. Freezing was extremely rapid to ensure that the material remained homogeneous. The coil with the frozen material attached was lifted out of the container and transferred to a vessel containing a large outer coil through which brine was passed at −50°. Brine at −23° was now passed through the inner coil. A high vacuum was applied and the water vapour condensed and was frozen on to the outer coil. When the material held on to the inner coil was completely dried it was transferred to a desiccator containing P_2O_5 and stored *in vacuo*. The fundamental difference between this process and the Flosdorf & Mudd (1935) process lies in the fact that in the latter at a certain stage the heat lost by evaporation is less than that entering by radiation and conduction so that in the terminal stages the temperature in certain parts of the material being dried is bound to rise. In the McFarlane process this does not occur as a constant low temperature is maintained throughout by refrigeration.

Estimation of percentage survival

Viable counts were carried out on the undried and dried drops. Usually ten drops were pooled, the mean count/drop was determined and the percentage survival calculated. The surface viable count method modified from that described by Miles & Misra (1938) was used. Under carefully standardized conditions and when adequate numbers of colonies were counted this gave results with a high degree of accuracy.

RESULTS

The effect of different suspending media and of reducing substances on survival rate; drying over P_2O_5 in vacuo

Melted nutrient gelatin was first selected as a suspending medium since it was thought that the gelatin and other substances present in the medium might coat the organisms protectively during drying. One-quarter strength Ringer solution was used for comparison. Ascorbic acid was also tested as it was considered that the death of organisms during drying and storage might in part be due to oxidation. *Salmonella paratyphi* B was used as the test organism.

Table 1. *Showing the percentage survival of* Salmonella paratyphi *B* (*Netherne*) *dried in melted* 10% *nutrient gelatin and in one-quarter strength Ringer solution, with and without the addition of ascorbic acid*

	Dried 3 days	Stored 23 days	
		In desiccator	On bench
Nutrient gelatin + ascorbic acid 0·1 %	53·6	35·7	17·0
Nutrient gelatin − ascorbic acid	32·1	2·8	2·7
Ringer solution + ascorbic acid 0·1 %	5·7	1·3	0·01
Ringer solution − ascorbic acid	4·4	0·003	0·001

It will be seen (Table 1) that *Salm. paratyphi* B was more effectively preserved in 10% nutrient gelatin than in one-quarter strength Ringer solution, and that ascorbic acid was also beneficial, particularly on storage. The drop in survival rate was, as would be expected, less rapid in the desiccator at room temperature than on the bench. The results with other suspending media and with glutathione as a reducing agent are shown in Table 2. Nutrient gelatin was better than horse blood, serum, mucin or polyvinyl alcohol under the conditions of test. Furthermore, glutathione in the concentration used did not exert the beneficial action of ascorbic acid. Although these substances might have been investigated further and other suspending media and reducing agents tested, in view of the favourable results with nutrient gelatin and ascorbic acid it was decided to confine the study to these substances.

Fig. 1 shows the effect of varying the concentration of ascorbic acid from 0·1 to 2·5% and of using nutrient gelatin diluted 1:10 with distilled water as suspending medium. The test organism was *Chromobacterium prodigiosum.*

Table 2. *Showing the effect of drying different organisms in melted* 10% *nutrient gelatin, nutrient gelatin plus heated rabbit serum, defibrinated horse blood, mucin, or polyvinyl alcohol and the effect of adding ascorbic acid and glutathione*

Species	Concentration of cells before drying	Concentration of reducing agent (%)	Suspending medium	Time of drying	Percentage survival
Salm. paratyphi B	$4 \cdot 0 \times 10^{10}$/ml.	Asc. acid 0·1	Defibrinated horse blood	3 days	11·25
		Glutathione 0·1	Defibrinated horse blood		7·5
		Nil	Defibrinated horse blood		10·0
		Asc. acid 0·1	Nutrient gelatin		100·0
		Glutathione 0·1	Nutrient gelatin		14·7
		Nil	Nutrient gelatin		15·7
Shig. shigae (K 624)	$1 \cdot 0 \times 10^{9}$/drop	Asc. acid 0·5	Nutrient gelatin	24 hr.	81·6
		Asc. acid 0·5	Nutrient gelatin 1 part + 9 parts heated rabbit serum		30·1
		Asc. acid 0·5	Nutrient gelatin 1 part + 99 parts heated rabbit serum		23·5
Bact. coli	$1 \cdot 2 \times 10^{9}$/drop	Asc. acid 0·25	Nutrient gelatin	2 days	40·5
	$1 \cdot 6 \times 10^{9}$/drop	Asc. acid 0·25	Mucin 2·5 %		$<1 \cdot 0$
Salm. typhi (T 150)	$1 \cdot 73 \times 10^{9}$/drop	Asc. acid 0·25	Nutrient gelatin	24 hr.	19·1
	$1 \cdot 57 \times 10^{9}$/drop	Asc. acid 0·25	Polyvinyl alcohol 2·0 %		14·6

The percentage survival increased with the concentration of ascorbic acid up to 0·5% and thereafter fell off; decreasing the concentration of nutrient gelatin gave somewhat lower survival rates. In other tests solutions of gelatin in concentrations higher than 10% gave no better result, and, moreover, were difficult to use owing to their high viscosity. Similar curves were obtained with *Bacterium coli* and *Salmonella typhi-suis* (Fig. 2). The deleterious effect of higher concentrations of ascorbic acid might be due to excessive acidity, since the pH, as estimated roughly by indicator dyes, was 4·8–5·2 with 0·5%, and 4·4 with 1% ascorbic acid.

Tests were made on *Chromobacterium prodigiosum* in nutrient gelatin with varying concentrations of ascorbic acid in which part of each suspension was first neutralized to pH 6·8–7·0 with one or two drops of N-NaOH before drying (Fig. 8). As anticipated, neutralized ascorbic acid was not deleterious in the higher concentrations. This fact has also been noted by H. B. Naylor (personal communication). In this test, as with *Salmonella typhi-suis* (Fig. 2), the optimal concentration was 0·25%; this concentration was used in nearly all the storage tests described below. Recently, however, a similar comparison of the effect produced by ascorbic acid and neutralized ascorbic acid in concen-

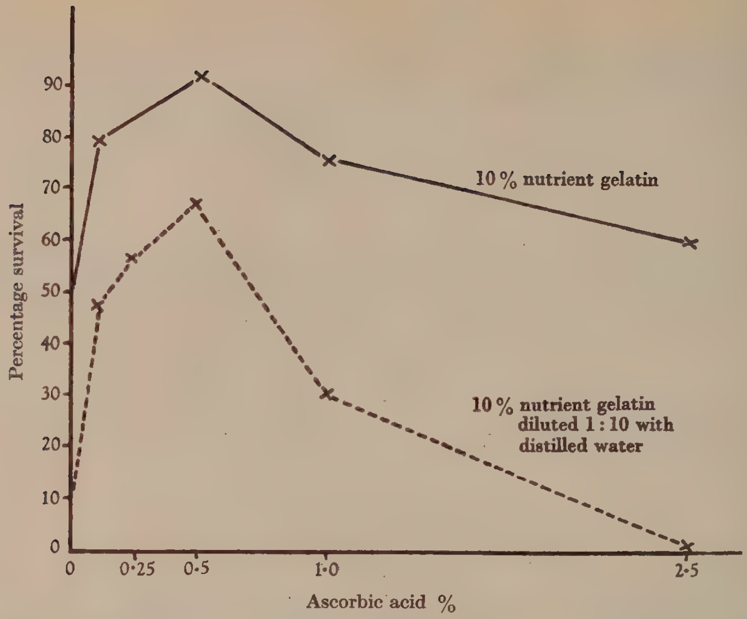

Fig. 1. The percentage survival of *Chr. prodigiosum* with varying concentrations of ascorbic acid after drying for 48 hr. in 10 % melted nutrient gelatin and in the same medium diluted 1 : 10 with distilled water (concentration of viable cells/undried drop $= 1{\cdot}7 \times 10^{10}$ approx.)

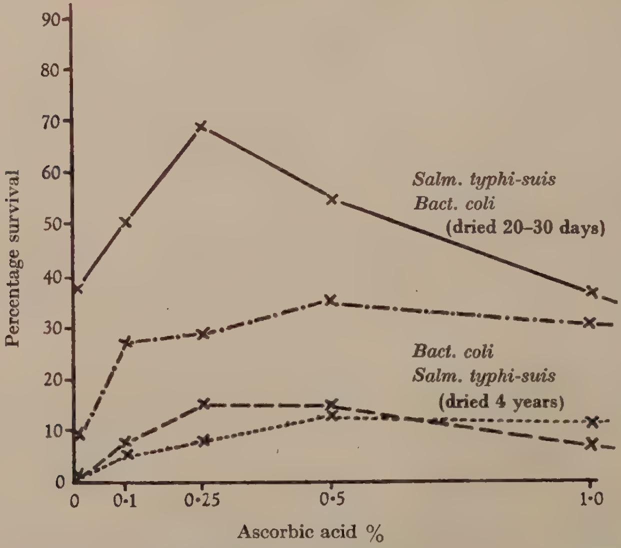

Fig. 2. The percentage survival of *Salm. typhi-suis* and *Bact. coli* with varying concentrations of ascorbic acid after drying for 3–4 weeks in melted 10 % nutrient gelatin, and after storage for 4 years. (Concentration of viable cells/undried drop $= 2{\cdot}2 \times 10^{8}$.)

trations up to 0·25% has been made with a number of different species, most of which were more delicate than those investigated earlier. The more important results are tabulated in Table 3.

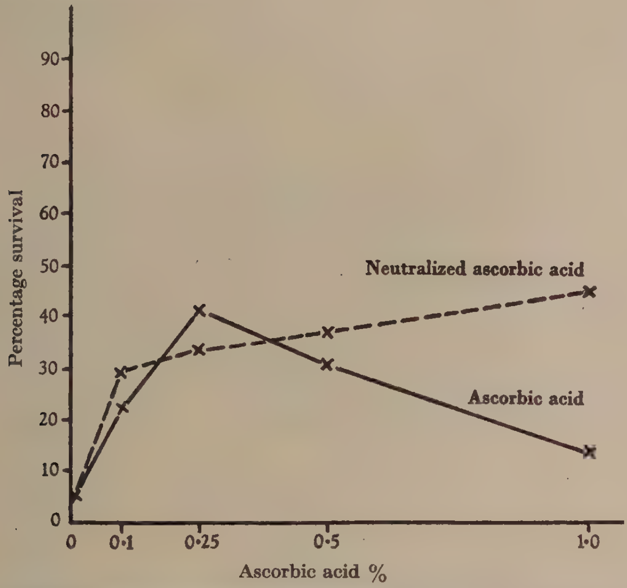

Fig. 3. The effect of neutralizing ascorbic acid with NaOH on survival rate of *Chr. prodigiosum* after drying for 9 days in melted 10% nutrient gelatin. (Concentration of viable cells/undried drop $=7{\cdot}3\times10^8$.)

Table 3. *Showing the percentage survival obtained on drying different species over* P_2O_5 *in melted nutrient gelatin containing varying concentrations of ascorbic acid, unneutralized and neutralized with* NaOH

Species	Average count per drop before drying	Days dried	Ascorbic acid %				
				Unneutralized		Neutralized	
			Nil	0·1	0·25	0·1	0·25
			Percentage survival				
C. diphtheriae gravis	$3{\cdot}2\times10^8$	2	44·0	61·1	78·0	61·7	58·7
Ery. rhusiopathiae	$3{\cdot}6\times10^7$	10	3·5	7·2	8·8	7·3	12·4
H. pertussis	$5{\cdot}2\times10^7$	4	11·0	11·5	1·0	25·3	21·0
H. influenzae	$3{\cdot}75\times10^8$	2	0·043	0·072	0·26	0·03	0·12
H. para-influenzae	$2{\cdot}34\times10^8$	3	0·027	0·26	0·38	0·081	0·66
Str. pneumoniae type I	$7{\cdot}78\times10^7$	2	3·5	1·76	1·54	4·1	7·1
Anaerobic *Streptococcus*	$2{\cdot}7\times10^6$	2	0·66	0·44	0·3	0·81	0·48
Cl. welchii	$4{\cdot}0\times10^7$	3	0·31	0·75	0·52	0·31	0·2
F. fusiformis	$2{\cdot}9\times10^5$	2	Nil	Nil	Nil	Nil	Nil
N. meningitidis	$1{\cdot}2\times10^7$	1	Nil	—	Nil	—	—

As might have been expected, the percentage survival values vary widely with the different species under test. With one species, *Corynebacterium diphtheriae*, the percentage survival rates are of the same order as those shown in Tables 1 and 2. With the exception of *Haemophilus pertussis*, the others are much less resistant to drying and two species, *Neisseria meningitidis* and *Fusiformis fusiformis*, failed altogether to grow on subculture. It should be noted that certain species, such as *Haemophilus pertussis* and *Streptococcus pneumoniae*, show an improvement only with neutralized ascorbic acid, presumably owing to the fact that they are particularly sensitive to acid. These results suggest that sodium ascorbate may be better than ascorbic acid for routine purposes.

A comparison of the slow drying process over P_2O_5 in vacuo *with the rapid freeze-drying process*

The relative merits of these two processes have not hitherto been investigated in much detail. *Chromobacterium prodigiosum* was suspended in 10% nutrient gelatin containing 0·5% ascorbic acid. In the first experiment test samples of the material were dried simultaneously by the rapid freeze-drying lyophile process of Flosdorf & Mudd and by the standard technique over P_2O_5 *in vacuo*. In the second test a comparison was made between the standard P_2O_5 method and the rapid freeze-drying process developed by McFarlane. The results are shown in Table 4.

Table 4. *Comparing the survival rates obtained by rapid freeze-drying processes with those given by drying over* P_2O_5 Chromobacterium prodigiosum *in 10% nutrient gelatin with ascorbic acid* 0·5%

Method of drying	Percentage survival
P_2O_5 drying—48 hr.	90·2, 86·1
Freeze-drying (Flosdorf & Mudd, 1938)	33·7, 29·6
P_2O_5 drying—48 hr.	60·7
Freeze-drying (A. S. McFarlane, 1942–3)	23·6

The survival values were approximately three times higher with P_2O_5 drying than with either method of lyophilization. As a result of these experiments and in view of its simplicity the slower drying method was adopted as a routine. It is, nevertheless, likely that with certain organisms, such as the meningococcus, lyophilization will prove to be the only satisfactory method, since (Table 3) *Neisseria meningitidis* failed to survive drying over P_2O_5 whereas it has been a general experience that it and the gonococcus will survive freeze-drying, according to some workers for as long as 18 years (Elser *et al.* 1935).

The higher survival rates obtained with *Chromobacterium prodigiosum* by the slower drying process might in part be accounted for by some degree of growth during drying. Tests with *Chr. prodigiosum* in nutrient gelatin showed that with the concentration employed (10^{10} organisms/ml.) the count might increase slightly during the first 3 hr. of drying. The maximum increase obtained was 30%, but it was usually less than this figure. In the presence of ascorbic acid this increase was never found to occur. The factor of growth therefore did not appear to play any substantial part in the results obtained.

In the experiments recorded in Table 4, the degree of drying by the different processes was measured. The weight of moisture removed by drying the drops to constant weight over P_2O_5 was 89·4% of that of undried material. By the McFarlane freeze-drying technique the loss of weight was 90·1% of the total. It would appear, therefore, that the method giving the highest degree of desiccation does not necessarily give the most efficient results in preserving viability.

The preservation of viability on drying by the gelatin-ascorbic acid process and on storing for long periods over P_2O_5

A large number of different bacterial species, and in one or two cases, a number of strains of the same species, were dried in melted nutrient gelatin + ascorbic acid over P_2O_5 and stored from 3·5 to 4·5 years. At the time these storage tests were set up the deleterious effect on an acid pH on certain species (Table 3) was not known, and nearly all tests were carried out with unneutralized ascorbic acid, usually in a concentration of 0·25%. The results are recorded in Table 5 and Fig. 4. It will be noted in Table 5 that the percentage survival immediately after drying varies from 90 to 12·5%, and after 4 years' storage from 50 to 1% with one exception, namely, *Vibrio cholerae*. Those species with the highest initial survival rate tended to die less rapidly than those with the lower initial survival rates. Thus the strains with an initial survival rate of 40% or over had in most cases fallen to about half their initial

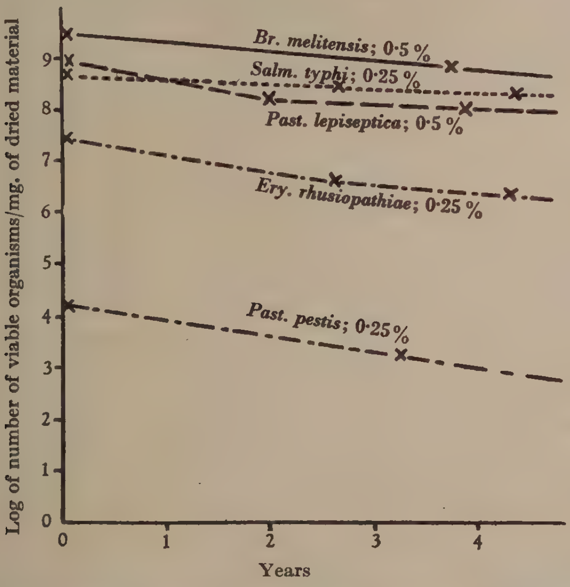

Fig. 4. The drop in viability of different species dried in melted 10 % nutrient gelatin with 0·25 or 0·5 % ascorbic acid and stored at room temperature over P_2O_5.

value in 2 years and to one-half to one-quarter in 4 years; for example, the majority of strains of Salmonellae, *Shigella shigae*, *Chromobacterium prodigiosum* and *Streptococcus pyogenes*.

Table 5. *Showing the survival rates of different species immediately after drying over* P_2O_5 *in melted nutrient gelatin with ascorbic acid and after long periods of storage at room temperature*

Species	Ascorbic acid (%)	Viable organisms per mg. of dried material, immediately after drying	Percentage survival immediately after drying (2–3 days)	Percentage survival after approx. 2 years' storage	Percentage survival after approx. 4 years' storage
Salm. typhi (T 150)	0·5	$6{\cdot}7 \times 10^8$	69·6	—	50·0
Salm. typhi (T 150)	0·25	$5{\cdot}5 \times 10^8$	75·0	34·7	21·8
Salm. typhi (T 135)	0·25	$6{\cdot}4 \times 10^8$	80·6	31·5	15·6
Salm. typhi (T 137)	0·25	$4{\cdot}0 \times 10^8$	48·5	—	18·8
Salm. typhi (T 142)	0·25	$2{\cdot}5 \times 10^8$	21·7	—	1·3
Salm. typhi (T 180)	0·25	$4{\cdot}6 \times 10^8$	48·9	—	16·7
Salm. paratyphi C	0·5	$8{\cdot}7 \times 10^8$	33·0	—	14·0
Salm. typhi-murium	0·25	—	—	20·0	18·4
Salm. typhi-suis	0·25	$8{\cdot}8 \times 10^7$	68·4	11·5	16·2
Salm. gallinarum	0·5	$6{\cdot}4 \times 10^7$	74·1	31·8	28·4
Bact. coli	0·5	$2{\cdot}3 \times 10^7$	25·5	—	12·0
Shig. shigae (K 624)	0·25	$2{\cdot}3 \times 10^8$	36·0	11·3	4·5
Shig. shigae (K 624)	0·5	$2{\cdot}9 \times 10^8$	22·9	—	4·2
Shig. shigae (S 19)	0·25	$6{\cdot}2 \times 10^7$	38·6	—	10·5
Vibrio cholerae (Inada S)	0·25	$3{\cdot}3 \times 10^5$	0·13	0·0	—
Vibrio cholerae (Inada O)	0·25	$1{\cdot}9 \times 10^5$	0·15	0·0	—
Chr. prodigiosum	0·5	$6{\cdot}0 \times 10^8$	71·3	26·5	16·0
Past. pestis (L 327)	0·25	$1{\cdot}6 \times 10^4$	—	—	1·0 (approx.)
Past. pestis (L 337)	0·25	$1{\cdot}6 \times 10^4$	—	—	1·0 (approx.)
Past. pestis (Schütze)	0·1	$2{\cdot}0 \times 10^3$	12·5	—	—
Past. lepiseptica	0·5	$1{\cdot}0 \times 10^9$	28·0	4·9	3·3
Br. melitensis (Mentone)	0·5	$2{\cdot}9 \times 10^9$	77·0	—	17·3
Ery. rhusiopathiae	0·25	$2{\cdot}6 \times 10^7$	28·3	4·5	2·6
Ery. rhusiopathiae	0·5	$2{\cdot}2 \times 10^7$	23·0	—	1·2
Strep. pyogenes (Richards)	0·0	$1{\cdot}14 \times 10^7$	68·6	12·6	8·6
Strep. pyogenes (Richards)	0·25	$9{\cdot}7 \times 10^6$	58·0	26·1	30·3
Strep. pyogenes (Richards)	0·25	10^6 approx.	—	44·0	31·4
Strep. pyogenes (Richards)	0·25	$1{\cdot}2 \times 10^5$	91·0	72·7	48·5
Strep. pyogenes (Richards)	0·25	10^4 approx.	—	62·0	65·7

Those with lower initial figures of 12–30% (as, for example, *Erysipelothrix rhusiopathiae* and *Pasteurella pestis*) have in most cases fallen to one-tenth to one-twentieth of the initial figure in 4 years. *Vibrio cholerae*, with the lowest initial survival rate recorded in this series (0·1%), failed to grow when tested a few months later. Different strains of the same species, i.e. *Salmonella typhi*, differed markedly in initial survival rates. The strains tested were all freshly isolated from fatal cases and were tested under identical conditions. The initial survival rates were 40% or over, but with one strain (T 142) it was about 20%. Other typhoid strains, e.g. Ty 2 and T1 (not included in Table 5), also gave lower figures, varying from 6 to 25%. An old laboratory stock strain, Ty Oxford, gave on a number of occasions initial survival values varying from 50 to 30%. It is evident that considerable variation in resistance to drying exists among different strains of typhoid bacilli, and the same may well be true for other species.

An indication of the effect of varying the cell concentration is seen with *Streptococcus pyogenes*. With a diminution in cell concentration, the percentage survival rate rises. A similar effect has been noted with *Pasteurella pestis* and *Salmonella typhi-murium*. The advantage gained in this way, however, is not marked and in order to obtain as high a number as possible of viable organisms in the dried material it is necessary to use highly concentrated suspensions. Fig. 4 shows the large numbers of organisms surviving in the dried samples over a 4-year period. It indicates that with a number of species, positive subcultures may be expected for a very long further period if the same conditions of storage are maintained.

The preservation of virulence

A number of pathogenic species, included in the above series, were tested for virulence before drying, and after drying and storing for 4 years (Table 6). Though the tests were incomplete and in most cases on a small scale there is some indication of the degree to which virulence has been maintained. Virulence has on the whole been extremely well preserved with the six species tested, which include some which are notoriously difficult to maintain in a virulent state. The results with the Pasteurella group are particularly satisfactory. The two typhoid strains were also tested for Vi-antigen by agglutination after storage and both agglutinated to titre with a pure Vi-antiserum.

DISCUSSION

The experiments provide a considerable amount of information about the factors which influence survival of bacteria on drying, measured in terms of the percentage survival rates estimated by a series of viable counts of the cultures tested.

In the first place the medium is important. Of the different suspending media tested melted nutrient gelatin was the most satisfactory. The constituents of this medium, namely, gelatin, Lemco beef extract and peptone, were not tested separately in any detail, but there is some evidence that the beneficial effect is mainly due to the gelatin (H. B. Naylor, personal communication).

A favourable effect on the survival of many species was also produced by ascorbic acid in certain concentrations. Cysteine and thiourea are said to produce a similar effect (H. B. Naylor, personal communication). These substances presumably counteract oxidation harmful to survival. Glutathione, however, in a single experiment was found to be inactive. Above concentrations of 0·25–0·5% the favourable effect of ascorbic acid is more than counterbalanced by the inimical effects of acid pH unless the acid be neutralized.

Table 6. *Showing the results of virulence tests on a number of pathogenic species before drying and after drying and storing for approximately four years*

Species	Observation period after infection (days)	Virulence test before drying		Virulence test after drying and storing for about 4 years	
		Infecting dose	Animals killed	Infecting dose	Animals killed
Past. pestis	14	0·5 ml. of 10^{-7} dilution of blood-broth culture, i.p.	Mice killed regularly	0·5 ml. of 10^{-7} dilution of blood-broth culture, i.p.	0/10 mice
				Do. 10^{-6} dilution	10/10 mice
Past. lepiseptica	7	100 approx., i.v.	3/6 rabbits	300 approx., i.v.	2/6 rabbits
				3000 approx., i.v.	6/6 rabbits
Ery. rhusiopathiae	11	0·5 ml. of 10^{-5} dilution of broth culture, i.p.	5/5 mice	0·5 ml. of 10^{-4} dilution of broth culture, i.p.	6/10 mice
Strep. pyogenes (Richards)	10	10,000 clumps approx., i.p.	27/30 mice	10,000 clumps approx., i.p.	9/10 mice
Salm. typhi (T 135)	7	9×10^7, i.p.	5/5 mice	$2{\cdot}2 \times 10^8$, i.p.	5/5 mice
Salm. typhi (T 150)		$1{\cdot}5 \times 10^8$, i.p.	5/10 mice	$2{\cdot}0 \times 10^8$, i.p.	1/5 mice
Salm. typhimurium	21	$1{\cdot}1 \times 10^5$, i.p.	5/6 mice	$4{\cdot}6 \times 10^5$, s.c.	9/10 mice
		$1{\cdot}1 \times 10^4$, i.p.	4/6 mice	$4{\cdot}6 \times 10^4$, s.c.	7/10 mice
		$1{\cdot}1 \times 10^3$, i.p.	4/6 mice	$4{\cdot}6 \times 10^3$, s.c.	5/10 mice
		$1{\cdot}1 \times 10^2$, i.p.	3/6 mice		

i.p. = intraperitoneal route; i.v. = intravenous route; s.c. = subcutaneous route.

With some particularly acid-sensitive species, e.g. *Streptococcus pneumoniae* and *Haemophilus pertussis*, even concentrations of 0·1–0·25% are deleterious, and an improvement in survival rate has only been brought about with neutralized ascorbic acid. It has yet to be determined whether sodium ascorbate is as effective as ascorbic acid in preserving viability on storage over long periods. With many species the survival rate with ascorbic acid in optimal concentration is 2–4 times that of control suspensions immediately after drying and from two hundred to ten thousandfold after storage for 4 years. With a few species, e.g. *Streptococcus pyogenes*, little or no improvement was apparent immediately after drying. After storage for 4 years, however, there was a tenfold advantage in the presence of ascorbic acid.

Another factor of importance is the method of drying. Slow drying over

P_2O_5 gave better results than freeze-drying when a direct comparison was made with *Chromobacterium prodigiosum* as test organism suspended in gelatin and ascorbic acid. Similar comparisons are required with other species. Slow drying proved unsatisfactory for preserving a few species such as *Neisseria meningitidis*, *Vibrio cholerae* and *Fusiformis fusiformis*. On the other hand, Herta Schwabacher (personal communication) found that eight strains of *F. fusiformis* survived slow drying in gelatin ascorbic acid. Six of these strains had previously failed to survive freeze-drying. *Neisseria meningitidis* and *N. gonorrhoeae* were successfully preserved by freeze-drying (Elser *et al.* 1935). Results with these organisms might well be improved by using gelatin ascorbic acid as suspending medium. It is likely that with many organisms, particularly the more delicate ones, *ad hoc* modifications in the technique for the different species will greatly improve the survival rate.

Lyophilization gives a slightly higher degree of desiccation than does drying over P_2O_5. In the latter process the material is in the form of thin disks; it may be that in these the drying is not uniform throughout and that in the interior, where there may be more residual moisture, conditions are more favourable for survival. In addition to the factors considered above there are others which may influence bacterial survival on drying. Further studies are required of the effect of varying the preparation of cell suspensions, comparing growths of different ages on solid media, and in static and aerated fluid media. The nature of the gaseous environment and other physical conditions under which the cells are dried may also be of importance.

It is noteworthy that with the gelatin ascorbic acid P_2O_5 method, unlike others previously described, storage in a high vacuum appears to be unimportant. This may be due to the presence of reducing substances in the dried material. It was, however, found essential to maintain a completely dry atmosphere. An accurate comparison of the results of these drying experiments with those of other workers is difficult owing to the fact that in very few cases previously have percentage survival rates been estimated. Otten (1932), using saline as a suspending medium and drying over H_2SO_4, found the following survival rates immediately after drying: *Salmonella typhi* 2·5–5%; *Shigella shigae* 0·5% or less; *Vibrio cholerae* 0·05%. He also stated that 'pneumococci, gonococci and meningococci were far under 1% and the same is the case for most of the other pathogenic bacteria such as *Brucella melitensis*, *Br. abortus*, *Pasteurella pestis*, *Haemophilus influenzae* and *H. pertussis*'. He noted a considerable improvement when meat extract was used in place of saline as the suspending medium, but no figures were given.

In addition to viability and virulence, properties such as chromogenicity were well preserved. In general, with nearly all species the organisms appeared to be maintained in a healthy condition in the dried material, as judged by the prompt and uniform development of colonies when subcultured directly on to an optimal medium. The gelatin ascorbic acid method is likely to be of considerable value for the maintenance of stock cultures of most bacterial species, especially those liable to undergo dissociation, such as phase I strains of *Haemophilus pertussis* and Vi strains of *Salmonella typhi*. It may also be found possible to apply this process to the preservation of living vaccines of organisms like avirulent *Pasteurella pestis* (Otten, 1936, 1941; Robic & Minec, 1938) and *Brucella abortus* S19 (Haring, 1938, 1939; Haring & Traum, 1937, 1941; McEwen, 1941; Huddleson, 1942). The organisms might be dried in ampoules and the suspensions reconstituted as required by the addition of warm sterile diluent. The dosage required at different periods after drying could be determined from a graph, should the fall in viability be found to be consistent under standardized conditions of storage.

The freeze-drying experiments were carried out in collaboration with Dr D. W. Henderson and Dr A. S. McFarlane. My thanks are due to the numerous persons who kindly supplied me with many of the strains used. I am indebted to the Chief Scientist, Ministry of Supply, for permission to publish this work.

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ADDENDUM

At the time of going to press H. B. Naylor & P. A. Smith have published a paper (1946, *J. Bact.* **52**, 565) on the drying of *Serratia marcescens* (*Chromobacterium prodigiosum*) which embodies the personal communications of H. B. Naylor referred to in the text. The greater part of the data presented above was available to these workers, who found that gelatin could be replaced by dextrin, pectin or Marmite without affecting the survival rate. Very high survival rates were obtained when the organisms from 24 hr. aerated cultures were suspended in a solution containing ascorbic acid, thiourea, ammonium chloride and dextrin at pH 6–7 and dried by lyophilization.

Observations on two very large Bacteria, *Caryophanon latum* Peshkoff and *Lineola longa* (nomen provisorium)

By E. G. PRINGSHEIM and C. F. ROBINOW

Botany School, University of Cambridge and Strangeways Research Laboratory, Cambridge

SUMMARY: The organism *Caryophanon latum* Peshkoff, a very large, Gram-negative, peritrichously flagellated bacterium of unusual structural complexity, which Peshkoff (1940) found in cow dung near Moscow, has been isolated in pure culture from Cambridgeshire cow dung.

The organism grows only poorly on routine nutrient agar, but thrives on yeast-extract + meat-extract agar, especially if supplemented with small amounts of sodium acetate. In liquid media it does not multiply, but very small concentrations of agar are sufficient to enable it to grow.

Individual cells of *C. latum* have a diameter of 2·5–3·2 μ. and are 10–30 μ. long or longer. They divide by binary fission like ordinary bacteria but have in reality a chambered, many-celled structure which gives the organisms a superficial resemblance to filamentous Cyanophyceae. The cell units are either discoid or shortly cylindrical and contain configurations of Feulgen-positive chromatinic bodies, similar to, but more complex than, those found in ordinary bacteria. We regard them as nuclear structures.

The provisional name of *Lineola longa* is proposed for another very large, Gram-negative, filamentous bacterium of unusual morphology, very different from *Caryophanon latum*, also found in cow dung. The new organism is peritrichously flagellated and grows in filaments, 1·5 μ. wide and up to 200 μ. long. The component cells of the filaments are few in number and exceedingly long, often 20–50 μ. Fragmentation into smaller units, which is the usual fate of filamentous forms in ageing cultures of ordinary bacteria, was never observed in *L. longa*.

In 1940 Peshkoff described *Caryophanon latum*, a new, very large bacterium distinguished by a high degree of structural differentiation.

Peshkoff first found this extraordinary non-sporing, Gram-negative bacterium in fresh cow dung gathered in the neighbourhood of Moscow. Pure cultures were obtained by plating samples which had been diluted with 2–3 parts of water and allowed to stand for 15–20 hr. From the study of living material Peshkoff concluded that the long rod forms, which represent the natural growth habit of the new organism, 'are not divided into separate cells and represent closed tubes containing a varying number of nuclei and a common protoplasm'. In 1946 he elaborated his point of view further and published high-power photographs of living *C. latum* filaments.

We have repeated Peshkoff's observation on the occurrence of *C. latum* in dung and have isolated and reinvestigated the organism. During this work a second very large but otherwise very different Gram-negative bacterium, previously undescribed, was encountered. The investigation of the two microbes revealed that the variety of organization possible in bacteria is larger than is commonly assumed.

CARYOPHANON LATUM PESHKOFF

Habitat. The immediate success of our search for *Caryophanon latum* suggests a wide, perhaps ubiquitous, distribution of this organism in fresh cow dung. The failure of earlier investigators to discover such a large, common and actively motile organism may be attributed to the fact that old manure, so often investigated on account of its practical importance, does not contain *C. latum.*

Under ordinary conditions *C. latum* reaches the peak of its development in cow dung a day or two after the faeces have been dropped in the field. The organism also appeared in two out of four samples collected during the winter from the surface of very fresh cow droppings on clean straw in a well-kept byre where gross contamination from the soil could be ruled out. On the other hand, the organism failed to develop in any of four samples of the contents of the rectum of cows, collected at the slaughterhouse. Thus we have not been able to decide whether *C. latum* is present in the intestines of cattle, or whether it is a strictly saprophytic organism which finds in cow dung particularly favourable conditions for its multiplication.

Isolation. Peshkoff found *C. latum* in three out of seven samples of cow dung. He does not state whether the dung was collected in field or cow-shed. We looked for the organism in cow droppings from grass land in Cambridge and had no difficulty in finding it, without dilution or special preparation of the dung, in five out of seven samples collected at the end of August 1945. In the next two months fourteen more samples from different localities were examined and nine of these were also rich in this species. In the following spring cow droppings collected in yet another place were again found to contain large numbers of the organism. Some samples contained smaller numbers than others, but since the period of development of the bacillus in dung is short, the number of motile organisms of characteristic appearance thereafter rapidly falling to zero, the best time for inspection may sometimes have been missed. Few or none of the large bacteria were found in samples of very fresh droppings, but the number usually increased greatly when the material was kept in its own moisture in closed glass jars at room temperature for a day or two. After this interval single loops-full of dung suspended in water were teeming with thousands of the actively motile rod form (Pl. 1, fig. 1).

To obtain a pure strain, a drop of a suspension of dung rich in *C. latum* was streaked on dung agar. Colonies of the large bacterium were visible, among those of other organisms, after 24 hr. In spite of their small size, it was not difficult to pick up individual colonies with a capillary pipette, and by repeated plating to obtain pure cultures. Other pure strains were obtained with greater ease by plating suspensions in which *C. latum* had been concentrated by fractional low-speed centrifugation. Five strains obtained in this way and on which the following description is based, were identified as *C. latum* Peshkoff. *C. tenue* Peshkoff (1940), a somewhat smaller organism, was never encountered in our material.

Cultivation. The dung agar used by Peshkoff was not a very satisfactory culture medium in our hands. Different dung samples varied greatly in growth-promoting power, and even when growth was good the bacteria were abnormally short, coiled and varied greatly in size. The addition of peptone, beef-extract or glucose and alterations of the pH proved to be either unfavourable to growth or without effect. On slightly alkaline nutrient agar (containing 0·5 % Bacto peptone and 0·5 % Bacto beef-extract) growth was much the same as on dung agar. This was contrary to the experience of Peshkoff, who found that *C. latum* did not grow on ordinary culture media. Very good growth was obtained on yeast-extract agar. Some batches of laboratory-made autolysates of baker's yeast gave excellent results, but others were unsatisfactory, and eventually we relied on the Difco product (Difco Laboratories Inc., Detroit, Michigan) in combination with either Bacto peptone (Difco) or Bacto beef-extract (Difco). Small amounts of sodium acetate had a specific growth-promoting effect in yeast-extract + beef-extract or yeast-extract + peptone media, giving heavier and more uniform growth than with any other combination of ordinary nutrients examined. A medium containing 0·50 % Bacto yeast-extract, 0·50 % Bacto peptone and 0·01 % sodium acetate, adjusted to pH 7·4–7·6, proved very favourable for isolation and maintenance.

In our cultures *C. latum*, which originally had a diameter of about 3 μ., has become somewhat smaller since its isolation some 8–12 months ago and now rarely exceeds 2·5 μ. in diameter. Migula (1897) reported the same experience with the originally very large *Bacillus oxalaticus*.

Growth on agar media. In 18 hr. at 26°, on the yeast-extract peptone acetate medium *C. latum* forms colourless, convex, circular, undulate colonies with an undulating edge, rarely exceeding 1–2 mm. in diameter, with a smooth or finely granular glistening surface. It grows in two forms of colony, exhibiting some of the characters of a rough or a smooth growth habit. Both may appear on first isolation. The smooth form predominates, but after some months in culture strains originally fully smooth may give rise to rough colonies, a phenomenon already noted by Peshkoff (1940). The smooth colonies consist of closely packed rods of fairly uniform length and have at first rather angular, jagged outlines (Pl. 1, fig. 2), but later become round or polygonal with smooth, unbroken contours. The rough colonies consist of long, loosely arranged and much folded chains which give a wavy, irregular outline to the colonies. The differences between smooth and rough colonies are more pronounced in young than in old cultures because after several days of growth the chains in the rough colonies break into short bacillary forms.

At the height of its development in cow dung the organism is a cylindrical, often slightly tapering, straight or gently curved rod, 10–30 μ. long and about 3 μ. wide, with rounded ends. The same dimensions are attained by *C. latum* in the periphery of young colonies on suitable media. Old cultures consist mainly of very short rods and single, more or less spherical cells (Pl. 4, figs. 11–13). In some samples of cow dung the rods were arranged end to end in chains of 200 μ. and longer.

The bacterium grows well at all temperatures from 20 to 37°; most of the observations described in this paper were made on cultures incubated for 18 hr. at 26°.

Colonies of recently isolated strains, after several days of growth on apparently favourable media, are liable to undergo a peculiar process of disintegration and are transformed into translucent patches consisting chiefly of empty cell walls and granular debris. The lysis first occurs in areas of confluent growth, and well-isolated colonies usually remain unaffected. Among the debris of lysed bacteria, structures resembling those in organisms of the pleuropneumonia group (the *L*-organisms of Klieneberger (Klieneberger & Smiles, 1942)) are very conspicuous. Small numbers of these elements, crisply staining vesicles with peripheral granules, were present after less than 24 hr. incubation in the colonies of all freshly isolated strains that we examined. It is still uncertain whether they represent a growth form of the bacterium or indicate the presence of a symbiont or parasite.

Growth in fluid media. According to Peshkoff, *C. latum* does not grow well in liquid media; this has also been our experience. Growth in dung extract was scanty, restricted to the surface and composed of deformed cells. Changing the pH of the extract and the addition of meat extract, peptone and glucose caused no improvement. Surprisingly enough the addition of very small amounts of agar (e.g. 0·1–0·2 %) to these liquid media stimulated growth considerably, although the cells were still abnormal in comparison with those grown on solid agar.

General morphology. C. latum is actively motile. Flagella stains and electron micrographs reveal a dense coat of peritrichous flagella whose diameter is approximately the same as that of the flagella of *Proteus vulgaris* and whose length is 9–12 μ. (i.e. three to four times the width of the organism). Curved individuals may convey the impression of a writhing, snake-like movement, but the organism is quite rigid and incapable of active change of shape. In agar cultures the bacterium often swims actively in the thin film of moisture covering and surrounding young colonies. It also slides quickly to and fro, at the same time rotating rapidly on its long axis where the wire loop has made shallow grooves in the surface of the medium. Prevented from moving about, e.g. by the pressure of a cover-glass placed on colonies growing on agar, many individuals, while remaining stationary, spin rapidly on their axes. These observations suggest that this bacterium would be a rewarding subject for a study of flagellar movement.

Apart from their large size and lively motility. the most conspicuous feature of the living organisms is a pattern of more or less regularly spaced, parallel, transverse lines which divide the long rods into a series of discoid compartments (Pl. 1, fig. 3). In optical sections of the bacteria, the transverse lines appear dark and the contents of the compartments bright. In organisms from young cultures continuous transverse lines are frequently found alternating with others that are interrupted by a central gap. Here and there particularly refractive compartments are seen, compressed into a biconcave shape by the adjoining normal ones (Pl. 1, fig. 3); their incidence increases greatly with the age of the cultures.

Peshkoff (1940, 1946) maintains that the clear sections of the filaments are nuclei (hence the derivation of the generic name), and that the lines between them represent the cytoplasm. It seemed more plausible to us to regard the relatively dark, parallel, transverse lines as cell *boundaries*, the clear sections between them as cytoplasm plus nuclear structures, and the biconcave, particularly refractile sections (not mentioned in Peshkoff's description) as dead compartments, compressed by the turgor of the adjoining cells. A study of fixed and stained preparations has confirmed our ideas.

The detailed structure of Caryophanon latum

Methods of fixation. The bacteria were fixed wet and while still on the agar medium. Squares cut from agar plates were exposed to osmic acid vapour for 1½–2 min. and inverted on cover-glasses; then lifted off, leaving an imprint of fixed bacteria on the glass. When the growth to be fixed was heavy the agar squares were pushed diagonally across the cover-slip, producing a rapidly drying smear. Fixed preparations were stored in 70 % ethanol. Comparison of fixed, unstained bacteria with living ones has shown that this method gives life-like fixation, and that the brief drying after fixation has no damaging effect on the cells.

Staining of cell walls. (1) Dried films of fixed bacteria were mordanted for 30 min. in 5 % tannic acid and then stained for 1 min. with 0·02 % crystal violet in water. In ordinary bacteria the cell wall and the cell membrane (i.e. the outer layer of the protoplast) are readily and differentially stained by this method (Robinow, 1945), but *C. latum* stains rather slowly. Clear-cut staining of the cross-walls in the interior of the large bacteria was sometimes only complete 12–24 hr. after the stained films had been mounted in water.

(2) Staining of the cell walls and cell membranes was achieved more quickly by flooding fixed and dried smears for 3–5 min. with Morton's flagella stain (tannic acid potassium alum night blue solution; Smith, 1905). In combination with this stain an orange light-filter was used.

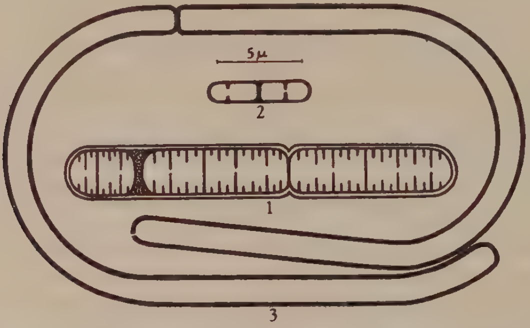
Staining of cell contents. Fixed preparations were stained for ½ hr. with Giemsa solution (1 drop/ml. of water) and differentiated under the microscope in water at approx. pH 5·5 (prepared by adding 2 or 3 loops of glacial acetic acid to a Petri dish of distilled water).

Staining of chromatinic structures. These were differentiated from the cytoplasm by treating fixed preparations for 7 min. with N-HCl at 56–60°, rinsing in tap water and distilled water and staining with Giemsa solution (Piekarski, 1937; Robinow, 1944, 1945; Vendrely & Lipardy, 1946; Vendrely, 1946), and differentiating with acetic acid water as above. The Feulgen reaction for chromatinic structures was carried out in the usual way. Following hydrolysis with N-HCl for 7 min. at exactly 60°, the preparations were left for 5 hr. in Schiff's reagent, rinsed in three changes of SO_2 water followed by 1 hr. in running tap water, counterstained with light green and mounted in DPX (British Drug Houses Ltd.).

Cellular structure of Caryophanon latum

Our idea of the cellular organization of *C. latum* is represented diagrammatically in Text-fig. 1. The systems of primary and secondary cross-walls, shown in the diagrams, are visible in the living organisms and inside the transparent shells of lysed filaments from old cultures (Pl. 3, fig. 10); they are also easily demonstrated in fixed specimens by both direct and indirect means. Indirect methods give the more impressive results and will be described first.

Basic dyes are much more firmly held by the protoplasts of *C. latum* than by its cell walls, and if staining in Giemsa solution or methylene blue is followed by brief differentiation in weakly acid water, the bacterial filaments are transformed into seemingly disjointed columns of deeply stained discoid or short cylindrical sections consisting of the cytoplasm and the nuclear structures, separated and bounded externally by colourless, faintly refractive membranes, i.e. outer cell wall and cross-walls. Secondary cross-walls projecting centripetally into the cytoplasm—in the manner familiar from studies of filamentous Cyanophyceae and certain bacteria (Robinow, 1945)—are demonstrated particularly clearly in this way (Pl. 2, fig. 5).



Text-fig. 1. Diagram to scale, illustrating the structural differences between *Caryophanon latum* (1), an ordinary bacterium (*B. cereus*) (2), and *Lineola longa* (3). Only the cell walls are shown; the cell contents and flagella have been omitted. The stippling of the biconcave section in *Caryophanon latum* indicates disintegration of the contents of one component cell.

The appearance is reversed with Morton's stain or tannic acid-crystal violet. These stains leave the cell contents almost colourless, while outer cell wall and transverse partitions in various stages of development are very distinctly stained and faithfully reflect the pattern of transverse lines which is so characteristic a feature of the living organism (Pl. 3, figs. 8, 9).

The refractive biconcave cells already referred to, which occur here and there among the normal discoid cells of living filaments, appear darker than normal cells in stained preparations (Pl. 3, fig. 8). They are cell chambers containing the remnants of disintegrated protoplasts.

Some of the cross-walls, usually the median ones, are continuous, but most have the shape of annular diaphragms, some with wide, others with narrow central apertures (Pl. 3, fig. 10). It would be incorrect, however, to assume on account of the apertures in the cross-walls that *C. latum* has a coenocytic structure. There is evidence that sister protoplasts are separated, presumably by the formation of cell membranes, before the closure of the central aperture in the cross-wall. This may be inferred from the readiness with which the bacterial filaments break up into their component protoplasts when placed in strong solutions of potassium nitrate or sodium chloride (Pl. 1, fig. 4), and in the variety of division stages to be found among the cells of the same filament.

The sectional construction of the bacteria is emphasized by the fact that disintegration of occasional protoplasts in the same filament, already referred to as giving rise to refractive, biconcave cells, is not accompanied by the dissolution of the rest of the filament. The number of concave cells per filament varies greatly, and the distribution of concave cells in the filaments is entirely at random.

This morphological picture is further complicated by the beginnings of tertiary cell walls which can often be detected arising from the outer cell wall of growing filaments and projecting into the cytoplasm in the narrow spaces between primary and secondary cross-walls, (Text-fig. 1). The simultaneous presence of cross-walls in three different stages of development—continuous, those with a central aperture and mere ridges on the inner surface of the outer cell-wall—gives a very close and remarkably regular striation which is best seen after fixation or in strong salt solutions. This pattern of transverse lines is shown in Pl. 2, figs. 6 and 7*a* and Pl. 3, fig. 8

Peshkoff's serial photomicrographs of growing rod forms of *C. latum* provide additional evidence that their component cells (or nuclei in the author's interpretation) divide independently. Provided the conditions of cultivation are optimal, however, the length of the rods is kept within narrow limits by the correlation of the rates of growth of the rod forms with their transverse fission. Fission takes place by gradual constriction in or near the middle region, in the plane of one of the preformed cross-walls. Secondary constrictions are often apparent in the middle region of daughter bacteria before the division of the parent organism is complete.

The chromatinic structures of Caryophanon latum

The interior of the bacterial cells is occupied by large chromatinic structures which are difficult to demonstrate by ordinary staining methods because they are largely obscured by the basophilic cytoplasm. These configurations are more clearly differentiated from the cytoplasm if staining is preceded by hydrolysis of the fixed organisms with N-HCl, a method which has already proved very useful in studies of the cytology of spores and vegetative cells of ordinary bacteria (Piekarski, 1937; v. Plotho, 1940; Robinow, 1944, 1945; Klieneberger-Nobel, 1945). Interpretation of the chromatinic structures is difficult, chiefly because in the narrow compartments into which the cell chambers are divided by developing secondary cross-walls, the chromatinic structures can only be viewed in profile. A better view is afforded by the hemispherical cells at the tips of the filaments, by the single spherical cells and the short two- or three-cell rods which abound in old cultures, and by the component cells of fixed and stained filaments disrupted by pressure. Comparatively favourable material for the study of the chromatinic structures is also provided by organisms fixed 1 or 2 hr. after transfer to fresh nutrient medium, in which more space is available for the chromatinic configurations than in the cells of organisms from older cultures.

Characteristic differences are found between the chromatinic structures in organisms fixed during the lag phase of multiplication following transfer, in cells from young growing colonies, and in cells from old cultures. During the resting stage the chromatinic matter is often arranged as a continuous ring, or in horseshoe fashion, around a disk of non-chromatinic material (Pl. 4, figs. 11–13) resembling, on a larger scale, the structure of the nuclei of resting bacterial spores. In growing cells the chromatinic structures appear to be composite and are more difficult to analyse (Pl. 4, fig. 14). Certain configurations, which were frequently seen, suggest that each group may be composed of several small, more or less rod-shaped bodies which multiply by longitudinal division (Pl. 4, fig. 15). Dividing chromatinic structures are numerous in the bacterial filaments from young cultures, but the detail of the process of multiplication is far less clear than it is in ordinary bacteria like *Bacterium coli* and *Bacillus cereus*. Division seems to coincide with, not to precede, the inward progress of developing cross-walls.

The chromatinic structures give a well defined, if not very intense, positive Feulgen reaction. As in ordinary bacteria, the chromatinic structures in *Caryophanon latum* are not only more intensely stained by Giemsa solution (after hydrolysis), but are also slightly larger than in Feulgen preparations. First emphasized by Piekarski (1937), this effect, has more recently also been noted by Wendler-Deane (1945) in *Plasmodium vivax* and *P. knowlesi*.

The presence of these structures in every cell at all stages of the growth cycle, their size and central position, as well as the fact that they are most conspicuous at times of most intense growth activities, indicate that they are important cell organelles. More specifically, the staining properties and the regular multiplication of the chromatinic structures suggest that they represent a true, though perhaps a primitive, nuclear apparatus.

Our photograph (Pl. 1, fig. 3) shows that the component protoplasts of living, unstained rods of *C. latum* appear brighter than the narrow partitions which separate them. This difference is accentuated by Peshkoff's 'double-negative' method of photography (1940). Photographs of living '*Achromobacter Epsteinii*' (Peshkoff, 1938) and of living *Proteus vulgaris* (which we have been able to examine through Dr Peshkoff's courtesy), in which bright configurations corresponding to the nuclear structures are clearly visible in the living bacteria, have induced Peshkoff to interpret the bright sections of the *C. latum* filaments in the same way. We are forced to disagree with this view, since the study of stained preparations has shown us that the bright sections in photographs of the living rods, Peshkoff's as well as ours, represent whole protoplasts, i.e. not only nuclei but also the cytoplasm which contains them.

In addition to the nuclear structures the cytoplasm usually contains varying numbers of small metachromatic granules which are conspicuous in fixed and stained cells not pretreated with HCl, but absent from the cytoplasm of hydrolysed bacteria.

A beaded condition of the cytoplasm was often encountered in the ridges flanking inwardly progressing cross-walls. It may have been this cytoplasmic granulation that has led Peshkoff (1946) to assume the existence of numerous ring chromosomes with distinct 'chromomeres' in the cells of *Caryophanon*. The true relationship of sets of the beaded ridges arising from the surface of the cytoplasm to the nuclear structures in the interior, is evident from a comparison of figs. 7*a* and *b* of Pl. 2.

The taxonomic position of Caryophanon

The general appearance of the organism and the character of the nuclear structures suggested to Peshkoff (1940) the existence of a 'direct relationship to the blue-green algae'. We do not agree with this hypothesis, but it must be admitted that many features of *Caryophanon*—the filamentous growth habit, the discoid cells, the occasional occurrence among them of empty, concave 'necridia' and the formation of hormogonia in the fragmentation of long filaments in old cultures—are reminiscent of certain Cyanophyceae, e.g. species of *Oscillatoria*. This similarity is, however, insufficient to establish a true taxonomic relationship.

Much more convincing, in our opinion, is the evidence relating *Caryophanon* to the great group of true bacteria. The features of *Caryophanon* which suggest this designation are (1) motility due to the presence of typical, peritrichate flagella; (2) regular binary fission of the composite rods; (3) the absence of a distinctive slime layer. The fact remains, nevertheless, that in *Caryophanon* one encounters structural features previously unknown in bacteria. To mention but one and perhaps the most obvious of these, the individual, which behaves in a manner analogous to a paucicellular rod form of, say *Bact. coli*, is really a many-celled filament (trichome).

Rod forms composed of more than one cell, it is true, are common in young cultures of many ordinary bacteria, and filamentous forms are well known in many species, but the former divide at the two- or four-cell stage and the latter do not behave as units of multiplication and are but transient stages in the growth cycle, followed by fragmentation or spore formation.

C. latum has much in common with some of the relatively large filamentous micro-organisms which have repeatedly been described as occurring in the intestines of various herbivorous mammals and which have in the past commonly been regarded as colourless blue-green algae. One of these, *Oscillospira guilliermondi* Chatton & Perard (*Oscillaria caviae* Simons), an inhabitant of the intestines of the guinea-pig, we have begun to investigate. Though exceeding *C. latum* in size, *O. guilliermondi* resembles that organism very closely in general appearance and construction, is motile by means of peritrichate flagella (Robinow, unpublished), and has the same kind of discoid cells and chromatinic structures. Pure culture studies on other members of this group are needed before the classification of these bacteria can be profitably undertaken.

LINEOLA LONGA (nomen provisorium)

In the multitude of micro-organisms seen in cow dung infusions another filamentous bacterium attracted our attention by its motility, combined with very great length and more than ordinary width. In its morphology and cytology it is so different from ordinary bacteria and also from *C. latum* that we propose to compare it with the latter. It will be described more fully in a separate paper and is here referred to under the provisional name *Lineola longa.*

The filaments formed by this bacterium are not as wide as those of *C. latum* but usually much longer. They are sufficiently tough and flexible to withstand much twisting and bending. Even the very long filaments are motile by means of peritrichous flagella. Like *C. latum*, *L. longa* does not actively alter its shape, and the seemingly writhing, snake-like character of its ponderous swimming movements is due to curvatures of the long filaments and to the rotation on their axis.

The filament diameter, 1·5–2 μ., does not exceed that of, for example, the rod forms in young cultures of *Bacillus megatherium*; it is the length that is extraordinary. Filaments 30–50 μ. long are common in agar cultures, and much greater lengths, up to 200 μ., are attained in liquid media. Cross-walls observable after tannic acid + crystal violet staining, occur at irregular intervals and are few and far between (Pl. 5, fig. 16). Filaments more than 50 μ. long consist of several sections; in shorter forms it is rare to find more than one cross-wall per filament. Staining with Giemsa solution reveals the absence of further subdivisions between the cross-walls and shows that filaments are built up of a small number of cells.

L. longa contains large numbers of small, rod- or dumb-bell-shaped Feulgen-positive bodies, similar in arrangement and appearance to those which Klieneberger-Nobel (1945) demonstrated in *Clostridium oedematiens* var. *gigas*. Attempts to stain these bodies by the HCl-Giemsa method have not given convincing results, and detailed observations on these structures have not yet been possible. Multiplication is by transverse fission in the plane of a preformed cross-wall. Often, but by no means always, fission divides a filament into halves.

DISCUSSION

C. latum and *L. longa* have certain general characters in common. They share a common habitat, they are both uncommonly large, peritrichously flagellated, Gram-negative and non-sporing, and they are difficult to define taxonomically. But they differ conspicuously in their construction. *C. latum* is closely subdivided into discoid cells and bears a superficial resemblance to filamentous Cyanophyceae. *L. longa* lacks subdivision into mononucleate compartments and, apart from the length of its component cells, is built like an ordinary bacterium. It differs from the filamentous forms of the latter and, indeed, from *Caryophanon* in that the filamentous growth habit is its only form of existence, there being no stage in its growth cycle at which fragmentation into small units takes place.

We have pleasure in thanking Dr R. Y. Stanier for helpful suggestions and constructive criticism and Dr F. W. G. Lund for his assistance in searching for *Caryophanon* in the Lake District.

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EXPLANATION OF PLATES

Unless otherwise stated osmic acid vapour is the fixative used for stained preparations. All stained preparations were photographed mounted in water.

PLATE 1

Fig. 1. *Caryophanon latum* in cow dung after 4 days at room temperature. Nigrosin smear. × 650.

Fig. 2. Small colony of the smooth type on beef-extract + yeast-extract acetate agar (10:1) after 18 hr. at 26°. × 154.

Fig. 3. Edge of a living colony of *Caryophanon latum* incubated for 24 hr. at 26°. The filaments are closely subdivided by numerous cross-walls. The two refractile biconcave segments in the central filament are disintegrated cells. × 1700.

Fig. 4. Living, unstained filaments of *Caryophanon latum* plasmolysed in saturated solution of sodium chloride. × 2350.

PLATE 2

Fig. 5. Cellular structure of *Caryophanon latum* from a 24 hr. culture. Only the cell contents (cytoplasm plus nuclei) are stained. Outer cell wall and cross-walls are not stained, and the bacteria consequently appear as columns of disjointed, disk-shaped protoplasts. The peripheral notches are optical sections of grooves in the cytoplasm caused by developing secondary and tertiary cross-walls. Osmic acid, Giemsa. × 2700.

Fig. 6. Different stages of transverse fission of typical *C. latum* individuals resembling the growth habit of ordinary bacteria. Differences in the appearance of the filaments are due to their lying at different levels of focus. Thus, surface detail, i.e. a closely spaced transverse striation, is in sharp focus in the bacterium indicated by the arrow, whereas the unstained nuclear structures in the interior are in focus in others. Tannic acid, crystal violet. × 2700.

Figs. 7 *a*, *b*. Two photographs of the same group of filaments from a young culture. (*a*) Close striation of the cytoplasm caused by incipient and more or less fully formed cross-walls. The cytoplasm is granular. (*b*) The nuclear structures in the interior after treatment with HCl. Giemsa preparations. × 2700.

PLATE 3

The magnification of the photographs on this plate is indicated by the scale underneath fig. 8.

Fig. 8. Formation of 'hormogonia' by the fragmentation of long filaments in a culture 4 days old. Note the darkly stained biconcave disintegrated cell in the right half of the upper of the two long filaments. The pattern of primary and secondary cross-walls is very clear and emphasizes the life-like fixation obtained with osmic vapour. Mordanted with 5 % tannic acid, lightly stained with 0·02 % crystal violet.

Figs. 9 *a*, *b*. Two photographs of a group of filaments from a 4 hr. old culture of *Caryophanon latum*; stained first with night-blue-tannic acid, to show the cell boundaries (*a*) and afterwards treated with HCl and stained with Giemsa solution to show the cytoplasm and the chromatinic structures of the component cells of the same group of filaments (*b*).

Fig. 10. Empty transparent shells of a chain of *C. latum* individuals from a 6-week-old agar culture. The cell contents have lysed, but the outer cell wall and the cross-walls remain. Most of the cross-walls are not yet fully formed and are annular diaphragms.

PLATE 4

The magnification of the photographs on this plate is indicated by the scale alongside fig. 14.

Figs. 11–13. Flat nuclei with peripheral concentrations of chromatin in resting forms of *Caryophanon latum* from 4–5-day-old cultures. A single cell, spherical during life, is seen in the upper half of fig. 12. In the short cells of the composite individuals in figs. 11 and 13 most of the nuclei are seen sideways; a fuller view of them is obtained in some of the more or less hemispherical end-cells.

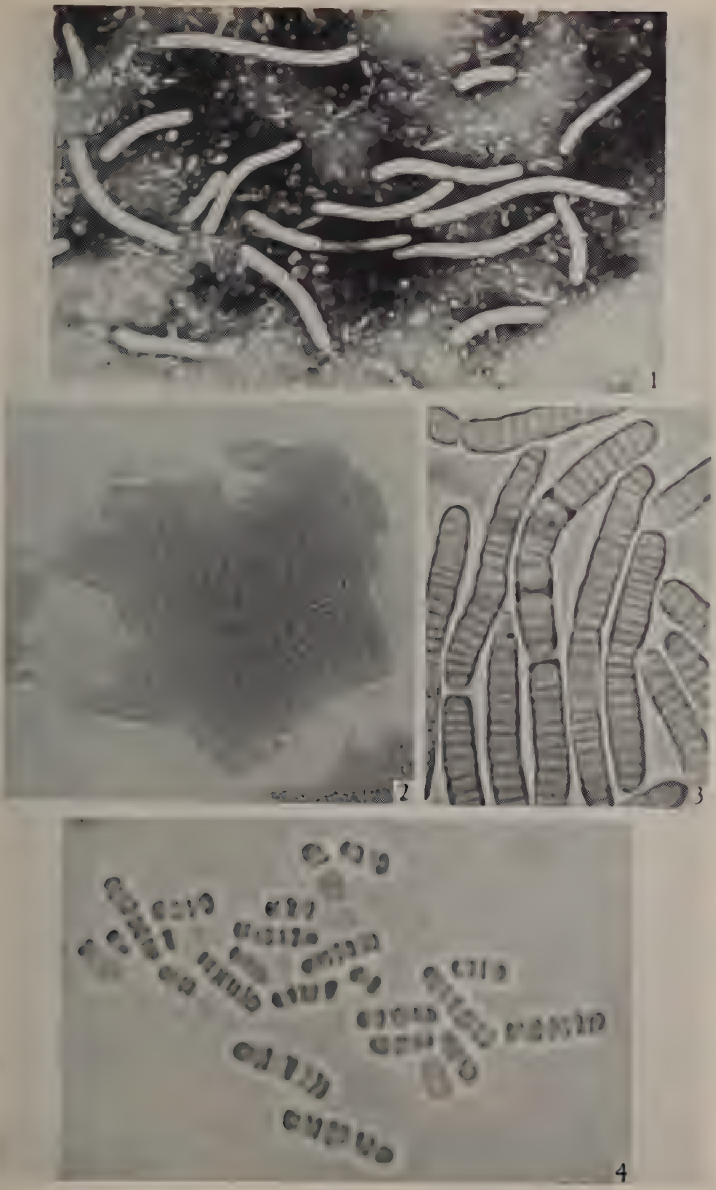
Fig. 14. Compact, polygonal, chromatinic structures in growing rod forms of *Caryophanon latum*, fixed 1½ hr. after transfer from an old culture to fresh nutrient medium.

Fig. 15. Chromatinic structures in growing filaments fixed 5 hr. after transfer from an old culture to fresh nutrient medium. Most of the chromatinic structures in the filament to which the arrow is pointing seem composed of groups of small dumb-bell-shaped rodlets.

PLATE 5

Fig. 16. *Lineola longa*. Osmic acid, tannic acid, crystal violet, smear preparation. × 3000.

(*Received* 23 *December* 1946)



Figs. 1–4

E. G. Pringsheim and C. F. Robinow—Observations on two very large bacteria.
Plate 1

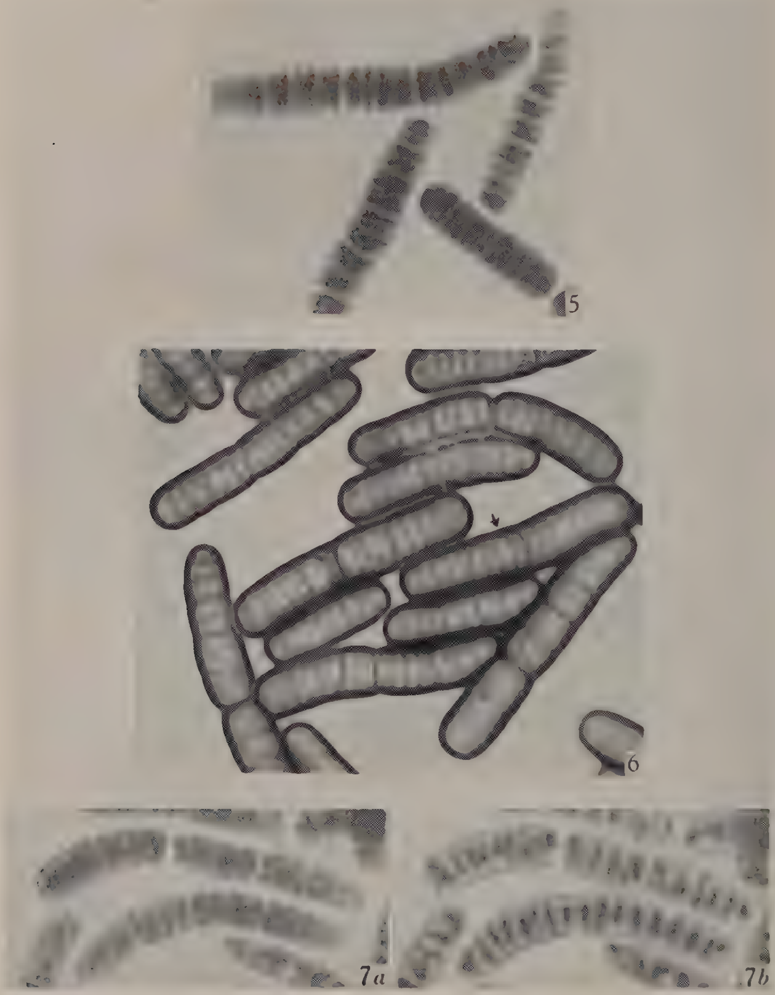


Fig. 5–7*b*

E. G. Pringsheim and C. F. Robinow—Observations on two very large bacteria.
Plate 2

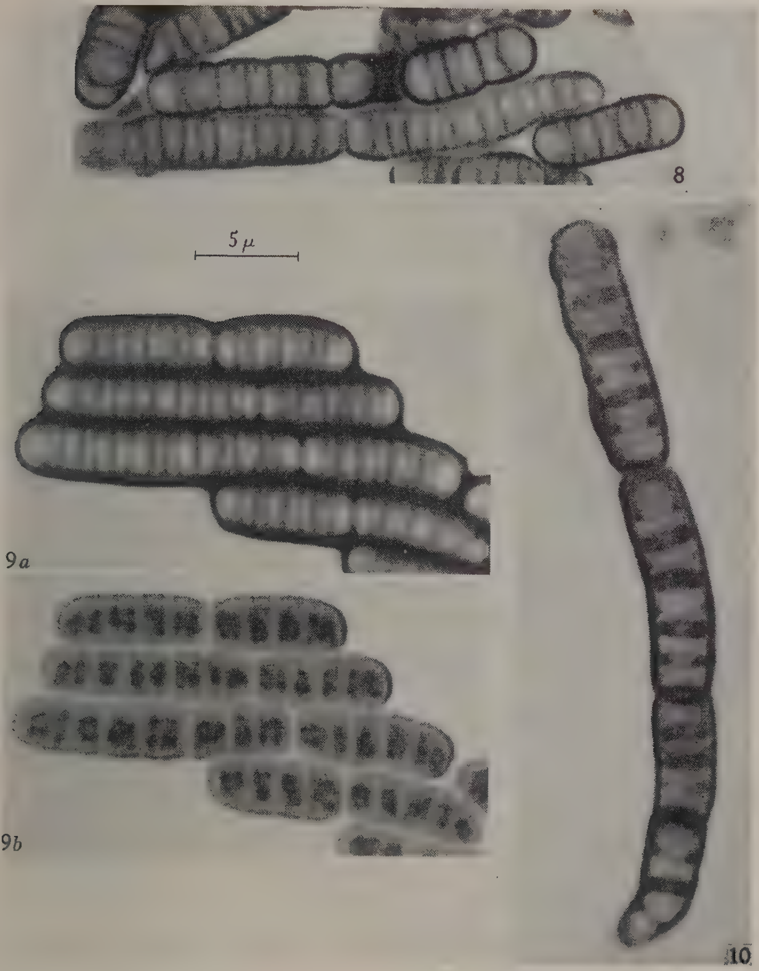
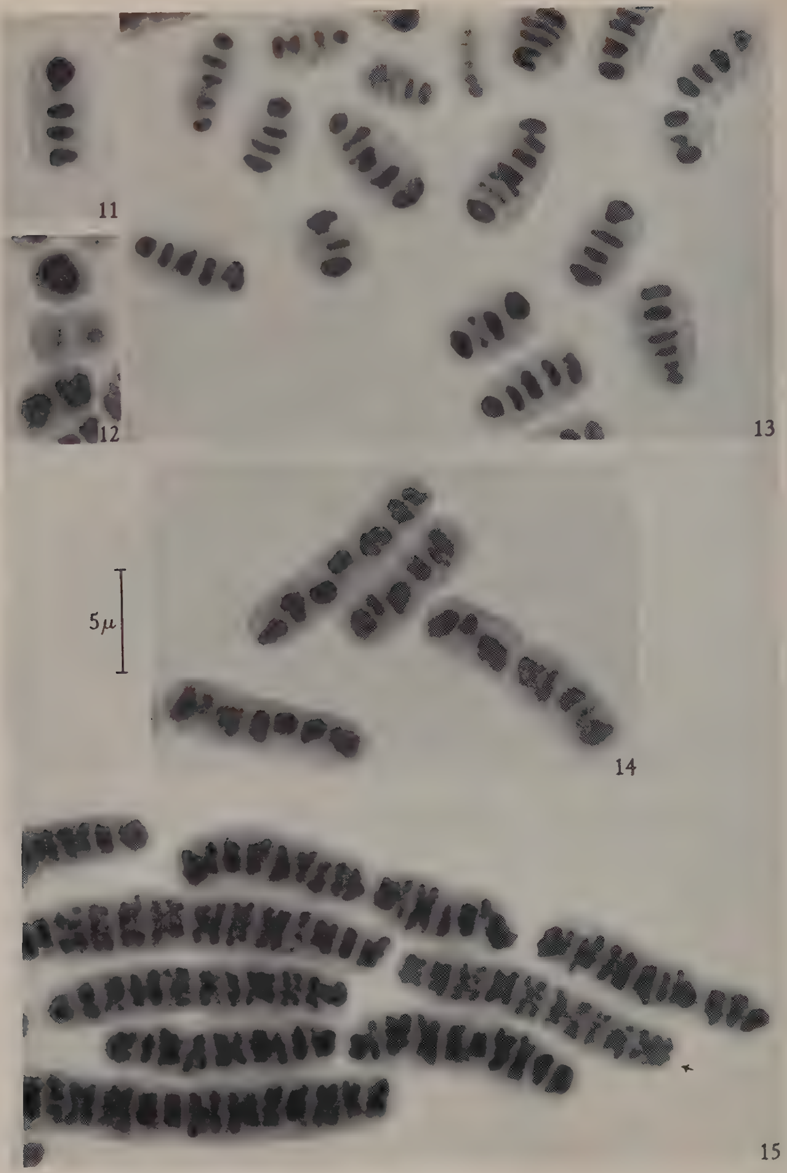


Fig. 8–10

E. G. Pringsheim and C. F. Robinow—Observations on two very large bacteria.
Plate 3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Figs. 11–15

E. G. PRINGSHEIM AND C. F. ROBINOW—OBSERVATIONS ON TWO VERY LARGE BACTERIA.
PLATE 4

Journal of General Microbiology, Vol. 1, No. 3

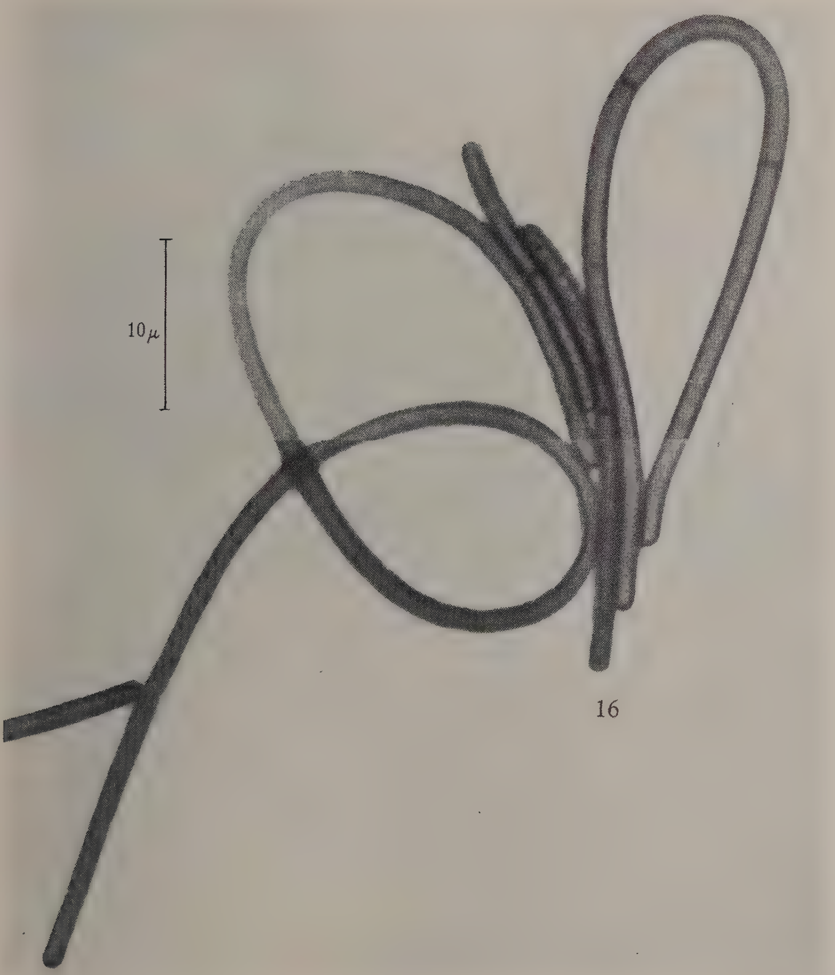

Fig. 16

E. G. Pringsheim and C. F. Robinow—Observations on two very large bacteria.
Plate 5

The Production of Acetylcholine by a Strain of *Lactobacillus plantarum*

By MARJORY STEPHENSON and ELIZABETH ROWATT

With an Addendum on the Isolation of Acetylcholine as a Salt of Hexanitrodiphenylamine

By K. HARRISON

The Medical Research Council Unit for Chemical Microbiology,
The Biochemical Laboratory, Cambridge

SUMMARY: A strain of *Lactobacillus plantarum* has been isolated from Sauerkraut which can produce acetylcholine during growth and in washed suspensions. The conditions necessary for the production of acetylcholine are: (i) the presence of choline and (ii) the simultaneous fermentation of carbohydrate. The acetylcholine is formed inside the cells and subsequently passes into the medium. The amount formed can be about 5 μg. acetylcholine/mg. dry wt. cells/hr. The acetylcholine was identified by the usual biological tests and was also isolated and identified as the salt of hexanitrodiphenylamine. The mechanism of the acetylation has not been discovered. Cells grown in the absence of added pantothenate acetylate extremely slowly. The rate is restored by the addition of pantothenate to the cell suspension.

Instances of the production of acetylcholine by micro-organisms are not very numerous. Ewins (1914) isolated it as the platinic chloride from 1600 ml. of a liquid extract of ergot prepared according to the *British Pharmacopoeia* and hence attributable to production by *Claviceps purpurea*; the yield was not given. Keil & Kritter (1934) found 40 μg./ml. acetylcholine in Sauerkraut juice. From 3 l. of Sauerkraut they prepared a reineckate of acetylcholine from which 0·2 g. of a gold chloride compound was obtained. The same workers (1935) obtained from fresh white cabbage some choline which they suggested was the origin of acetylcholine in Sauerkraut, but they were unable to find choline in fresh cucumber, though acetylcholine was found in fermented cucumber; they stated that choline added to fermenting cucumber did not increase the yield of acetylcholine, which finally amounted to 25 μg./ml. estimated biologically. The organism was described as a lactic acid bacillus.

Keil & Gropp (1934–5) obtained 0·012 g. of a gold salt of acetylcholine from 2·7 l. of fermented cucumber juice. Its isolation from a silage of maize and sunflowers has also been reported (Keil & Pörtner, 1935).

Lactic acid bacteria producing acetylcholine in the fermentation of plant juices seem widespread. They have been isolated from: Sauerkraut; fermenting cucumber silage (Habs, 1937–8); fourteen kinds of fermented vegetables (Möller & Ferdinand, 1937–8); rat gut, human mouth and faeces (Habs, 1937–8).

It is impossible to decide from the data whether strains isolated from all these sources represent the same organism. Where bacteriological identification has been attempted (Möller & Ferdinand, 1937–8; Habs, 1937–8), it seems that the organism is a lactic acid-producing bacillus, found most frequently in fermenting plant juices, having a temperature optimum below 37°.

In comparing the content of acetylcholine in fermented plant juices and in animal tissues the presence in the latter of an active choline esterase, an enzyme which has not so far been reported in the former, must be remembered. In animal tissues the spleen is among the richest in acetylcholine. Dale & Dudley (1929–30) extracted it from horse spleen with cold ethanol in amounts equivalent to 10 μg./g. wet tissue. From 32·34 kg. of spleen these workers obtained 64·5 mg. of acetylcholine as the dichloroplatinate. Chang & Gaddum (1933) estimated the acetylcholine content of a number of animal tissues and found the placenta and spleen to be the richest, containing about 30 μg./g. in both cases. Other tissues had only 1–4 μg./g. or less. Stedman & Stedman (1937) found 7 μg./g. of ox brain (wet weight), from 8 kg. of which they isolated it as the chloraurate. Thus fermented plant material yields acetylcholine in amounts greater than, but comparable with, spleen, i.e. about 60–80 μg./ml.

The present research was undertaken with the object of finding the origin of acetylcholine in fermented plant material and of studying its mode of production. As Keil's strain was not available to us it was necessary to isolate an acetylcholine-producing organism.

MATERIAL AND MEDIA

Sauerkraut

Sauerkraut was prepared as follows: About 1 kg. of finely shredded cabbage was put in a Kilner bottling jar with 20 g. NaCl in alternate layers, well pressed down and left at room temperature for 6–10 days; by this time the juice was at pH 3·8 and gave a positive reaction for acetylcholine (see below). At this stage the organism could be isolated; later the organisms tended to die off due to prolonged exposure to a low pH.

Cucumber juice

Fifty-one kg. of ridge cucumbers were peeled, minced and squeezed in a press; 25 l. of juice were obtained. This was filtered through pleated paper into 5 l. flasks and steamed for 3 hr. on three successive days. It was tubed and resterilized as required.

Media

Medium 1 (*glucose agar*). 330 ml. of a tryptic digest of casein (equivalent to 10 % casein); 10 ml. 10 % Marmite; 10 g. glucose; 30 g. agar; water to 1 l.

Medium 2 (*for heavy growth*). Inorganic medium (no. 3) 100 ml.; 330 ml. of a tryptic digest of casein (10 %); 10 ml. 10 % Marmite; 20 g. glucose (or other sugar); 10 ml. M-sodium acetate; 200 mg. cysteine HCl sterilized by filtration and added last; water to 1 l.

Medium 3. *Inorganic.* KH_2PO_4, 3·0 g.; $MgSO_4.7H_2O$, 2 g.; NaCl, 3 g.; $(NH_4)_2HPO_4$, 12 g.; $FeSO_4.7H_2O$, 0·03 g.; $CuSO_4.7H_2O$, 2·5 mg.; $MnSO_4.7H_2O$, 2·5 mg.; $ZnSO_4.7H_2O$, 2·5 mg.; water to 1 l.

Medium 4. *Synthetic.* Inorganic medium (no. 3) 100 ml.; glucose 20 g.; M-sodium acetate 100 ml.; *l*-asparagine 0·1 g.; *l*-glutamine 0·1 g.; *l*-cysteine 0·2 g.; *l*-tryptophan 0·02 g.; *l*-leucine 0·02 g.; *l*-isoleucine 0·02 g.; *l*-serine 0·02 g.; *l*-phenylalanine 0·02 g.; *l*-aspartic acid 0·02 g.; *l*-valine 0·02 g.; *l*-tyrosine 0·02 g.; *l*-alanine 0·02 g.;

l-threonine 0·02 g.; *l*-lysine 0·02 g.; *l*-methionine 0·02 g.; *l*-arginine 0·02 g.; *l*-histidine 0·02 g.; thymine 0·01 g.; adenine 0·01 g.; *l*-glutamic acid 0·02 g.; uracil 0·01 g.; hypoxanthine 0·01 g.; guanine 0·01 g.; to which were added the following growth factors: aneurin 200 μg.; riboflavin 200 μg.; calcium pantothenate 100 μg.; nicotinic acid amide 200 μg.; *p*-aminobenzoic acid 100 μg.; pyridoxin 100 μg.; folic acid concentrate equivalent to about 5 μg.; water to 1 l.

Medium 5. *Semisynthetic.* Inorganic medium (no. 3) 100 ml.; acid hydrolysate of casein (10 %) 100 ml.; glucose 20 g.; M-sodium acetate 100 ml.; *l*-tryptophan 200 mg.; cysteine HCl 200 mg.; growth factors as in medium 4; water to 1 l.

Medium 6. *Reaction solution* (does not support growth). McIlvaine's buffer pH 7·5 2 ml.; (M-sodium acetate 1 ml.*) 20 % glucose 1 ml.; 0·5 % choline HCl 1 ml.; ($MnSO_4 + MgSO_4$ each 0·4 % 1 ml.*) used with 1 ml. of a bacterial suspension about 20 mg./ml.; water to 10 ml. (* these items later found to be dispensable).

Neither the cucumber juice nor media 1–6 contained preformed acetylcholine.

Methods of estimation

Miscellaneous. The amount of growth and of cell suspension used were estimated by the use of a photoelectric turbidimeter, from the readings of which the dry weight was read off from a calibration curve constructed for the organism; results are recorded in terms of dry weight of cells throughout. Glucose was estimated by the method of Miller & Van Slyke (1936) and lactic acid by the methods of Friedemann, Cotonio & Shaffer (1927) and Friedemann & Kendall (1929), the latter after treatment with copper lime.

Volatile acids were estimated by the method of Friedemann (1938).

Acetylcholine. The method used was essentially that of Chang & Gaddum (1933), the exact procedure being that employed by Dr Feldberg, to whom we are indebted for instruction in using it. The procedure is as follows: The standard acetylcholine is prepared fresh each day and made up so as to contain 1 μg./ml. in slightly acid water.

Eserine stock solution consists of 1 g. eserine in 200 ml. of slightly acid water (0·002 N-HCl); 1 ml. of this solution is diluted afresh each day with 500 ml. frog Ringer.

The muscle is excised and put up in the glass chamber with frog Ringer and aerated with a steady stream of air bubbles for 1 hr.; the Ringer is then drained off and replaced by eserinized frog Ringer and aerated for a second hour. The muscle is then ready for use.

The standard acetylcholine and the solutions to be assayed should be kept in iced water. Acetylcholine standard solution (0·2–0·5 ml.) is made up to 10 ml. with eserinized frog Ringer and poured into the chamber and the muscle allowed to contract, the contraction being registered on a drum moving at about 10 mm./min. The period allowed for contraction is 1·5 min. The muscle is then washed once with frog Ringer and left at rest in eserinized frog Ringer for 5 min. The assays are repeated alternately with standard acetylcholine and the test solution till two adjacent contractions are obtained of the same height.

In order to eliminate error due to activating or sensitizing substances other than acetylcholine in the unknown solution, a portion of this should be boiled at pH 10, neutralized and a volume added to the acetylcholine standard equal to that used in the assay.

THE ORGANISM

Isolation

From the Sauerkraut prepared as described 1 ml. samples of juice were withdrawn at intervals and tested for acetylcholine. The test was positive on the 6th day, when the acetylcholine amounted to 2·6 μg./ml. The juice was then heavily inoculated into cucumber juice which was incubated at 25° and tested for acetylcholine daily; a positive reaction was obtained on the 4th day when the concentration was *c.* 1·2 μg./ml. The culture was again subcultivated into cucumber juice and plated on to cucumber juice agar; two types of colony were obtained: large (*A*), and small (*B*); both were replated on to glucose agar (medium 1) plates; *A*-type colonies appeared in 20 hr. and *B*-type in 40 hr.; *A* organisms were Gram-positive streptococci and *B* organisms were Gram-positive short rods; when subcultivated into cucumber juice *A* gave no acetylcholine and *B* gave acetylcholine in 5 days.

Characteristics

The organism was kindly examined for us by Dr P. M. F. Shattock of the National Institute for Research in Dairying, Shinfield, near Reading, to whom we owe the following details:

Morphology: short rods with rounded ends usually in pairs, Gram-positive. Grows well aerobically at 30°, will not grow at 45°; growth stimulated by yeast extract. Very slow production of acid in litmus milk. Acid from sucrose, lactose, maltose, raffinose, salicin and aesculin. No acid from xylose. Provisionally identified as *Lactobacillus plantarum.*

A culture has been deposited in the British National Collection of Type Cultures, no. 7220, and in the American Type Culture Collection, no. 10241.

Other organisms tested for the production of acetylcholine

Twenty recently isolated strains of streptococci were grown in cucumber juice for 4–6 days and the medium then tested for acetylcholine; none was found. The following strains of *Lactobacillus* were kindly supplied by Dr Shattock from the National Institute for Research in Dairying, and were tested on the same medium after 48 hr. growth: *Lb. bulgaricus*, *Lb. helveticus*, *Lb. acidophilus* (Booth), *Lb. delbrücki* 4033, *Lb. delbrücki B*, *Lb. casei* 3253, *Lb. casei* Y.C.T.I., *Lb. pentoaceticus* (107), *Lb. plantarum* 3254 and *Lb. plantarum* 4125; all these gave no acetylcholine. *Lb. odontolyticus* gave 4·5 μg. acetylcholine/ml.; *Lb. plantarum*, our strain, gave 7·0 μg./ml. The *Lb. odontolyticus* was subsequently identified as *Lb. plantarum.*

Whether our organism is identical with Keil's we have not enough data to decide, but the very different temperature optima for acetylcholine production, below 25° for our organism and 30–35° for Keil's (Möller & Ferdinand, 1937–8), suggest that they are distinct, though it must be admitted that the temperature optimum for growth or any chemical reaction is not a stable characteristic.

General biochemical characteristics

The organism is a facultative anaerobe, it has no detectable catalase, cytochrome or cytochrome oxidase. In accordance with its lack of haematin enzymes its respiration as measured by the manometric oxygen uptake was of a very low order, Q_{O_2} 6–10, and was not materially influenced by age of culture.

The anaerobic oxidation of glucose by methylene blue (MB) was of a higher order; the oxidation of sugars other than glucose was low or negligible (Table 1). When the organism was grown on the sugar in question, i.e. was adapted, the Q_{MB} (sugar) was higher (Table 2).

Table 1. *The oxidation of sugars by unadapted cells of* Lactobacillus plantarum *as measured by the reduction of methylene blue*

Growth medium 2 was used. Each Thunberg tube contained: 1·0 ml. bacterial suspension; 1·0 ml. phosphate buffer pH 6·5; 0·1 ml. 0·5 % methylene blue (MB); 1·0 ml. 0·5 % sugar or water; 1·8 ml. water. The end-point was read as 90 % reduction of the MB. $Q_{MB} = \mu l.\ O_2$ equivalent to MB reduced/mg. dry wt. cells/hr.

Sugar	Q_{MB} (sugar)	Q_{MB} (blank)
Glucose	50	2·8
Fructose	9·4	2·8
Maltose	28·1	9·4
Sucrose	9·7	6·8
Lactate	6·9	2·8

Table 2. *The oxidation of sugars by adaptea cells as measured by the reduction of methylene blue*

Cells grown on medium 2. Each Thunberg tube contained: 1·0 ml. bacterial suspension; 1·0 ml. phosphate buffer pH 6·5; 0·1 ml. 0·5 % methylene blue (MB); 1·0 ml. 0·5 % sugar or water; 1·8 ml. water. End point 90 % reduction of MB. $Q_{MB} = \mu l.\ O_2$ equivalent to MB reduced/mg. dry wt. cells/hr.

Sugar *A**	Q_{MB} (sugar)*	Q_{MB} (glucose)	Q_{MB} (blank)
Fructose	77	77	5·6
Galactose	71	79	14
Arabinose	6·9	35	2·5
Sucrose	25	73	3·4
Maltose	18·4	72	4·6

* Sugar to which organism was adapted.

The organism is more efficient as a fermenter than as an oxidizer. The fermentation was measured manometrically by the liberation of CO_2 by fermentation acid from bicarbonate buffer. The Q_{CO_2} of glucose increased with age of culture from Q_{CO_2} 120 for 20 hr., to Q_{CO_2} 191 for 44 hr. Fermentation of other sugars was low or negative with unadapted cells, but increased when the organism was grown on the sugar studied (adapted cells) (Table 3).

Temperature optimum for fermentation. The bacteria were grown for 2 days at 25° on medium 2. The fermentation was carried out in Thunberg tubes; each tube contained: 2·5 ml. phosphate buffer pH 7·0; 1 ml. bacterial suspension (2·5 mg./ml.); 1 ml. water; 0·5 ml. 0·5 % glucose in the hollow stopper. The tubes were evacuated and placed in the bath for 10 min. for temperature equilibration, the glucose was then tipped in. The tubes were removed at the end of 30 min. and the glucose estimated by the ceric sulphate method. The temperature optimum for fermentation is 40° (Fig. 1) and the pH optimum by the same technique 5·5–6·8 (Fig. 2).

Type of fermentation. The main product of fermentation was lactic acid, 71–83 % of the glucose fermented by washed suspensions of cells appearing as lactic acid; in these circumstances 20–30 % of the glucose was usually assimilated by the cells. Since it was of importance to be certain whether this organism did in fact produce volatile acids these were specifically looked for.

A mixture of cells (280 mg.), 250 ml. phosphate buffer pH 6·5 and 2·5 g. glucose was fermented to completion at 25°, NaOH being added at intervals to keep the pH approximately constant. The cells were then centrifuged off, the supernatant fluid filtered through Kieselguhr, made alkaline to phenolphthalein and evaporated *in vacuo* to 25 ml. Volatile acid was estimated in this volume by the method of Friedemann (1938). Volatile acid (6·46 mg. calculated as acetic acid) was obtained, equivalent to 0·35 % of the glucose fermented. This amount is within the limits of the experimental error and provides no evidence that acetic acid is a product of fermentation by this organism.

Table 3. *The fermentation of sugars by unadapted cells (grown on glucose) and by adapted cells (grown on the sugar subsequently fermented)*

Growth medium 2 used for cultivation of cells. Each manometric cup contained: 1·5 ml. $NaHCO_3$, pH 7·2; 1·0 ml. bacterial suspension about 8 mg./ml.; 0·5 ml. 0·4 % glucose (or water) in side bulb. Temperature 37°; gas phase 5% CO_2 in N_2. $Q_{CO_2} = \mu l.\ CO_2/mg.$ dry wt. cells/hr.

	Unadapted cells		Adapted cells		
Sugar	Q_{CO_2} (sugar)	Q_{CO_2} (blank)	Q_{CO_2} (sugar)	Q_{CO_2} (glucose) (for comparison)	Q_{CO_2} (blank)
Fructose	28·3	4·7	457	377	5·5
Galactose	2·0	1·0	226	198	1·9
Maltose	2·6	2·6	184	178	1·7
Sucrose	0	0	88	272	2·5
Lactose	0	2·6	309	329	0
Xylose	0	0	0	159	1·3
Arabinose	0	0	108	175	1·9
Hexosediphosphate	31	0	—	—	—

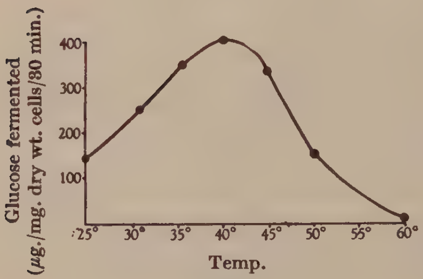

Fig. 1

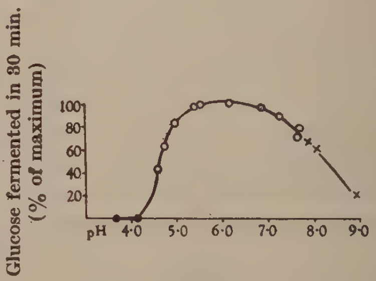

Fig. 2

Fig. 1. Effect of temperature on rate of fermentation of glucose.

Fig. 2. Effect of *p*H on rate of fermentation of glucose. ●—● acetate buffer; ○—○ McIlvaine's buffer; ×—× borate buffer.

THE PRODUCTION OF ACETYLCHOLINE

Formation during growth of the organism

So far acetylcholine had only been produced by organisms growing in vegetable juices; it is, however, produced on media 1 (liquid) and 2, and in small quantities occasionally on 5, the medium best suited for the production being a mixture of equal volumes of cucumber juice and medium 2. The organism grown for 20 hr. on 1100 ml. of this medium produced 28 mg. acetylcholine and 490 mg. dry wt. of cells, the acetylcholine thus produced amounting to 25 μg./ml. culture and 57 μg./mg. cells. The acetylcholine was distributed between the cells and the medium. The total culture (cells and medium) contained 24·7 μg. acetylcholine/ml., of which 12·3 μg./ml. was in the supernatant. The washed cells added immediately to the fluid in the chamber containing the muscle gave no response; when, however, the cell suspension was slightly acidified, boiled and neutralized, it gave with the muscle an immediate response equivalent to 9·6 μg. acetylcholine/ml.; this response was abolished by boiling at pH 10.

This result is reminiscent of the synthesis of an acetylcholine precursor by brain tissue reported by Mann, Tennenbaum & Quastel (1938), who effected its liberation by the action of acid and of chloroform. We are inclined to think that in the case we were studying the acetylcholine was free in the cell, since it was liberated by the action at room temperature of surface active agents such as tyrocidin, aerosol O.T. and also by disrupting the cells by shaking with glass beads (Curran & Evans, 1942).

Thus 1 ml. cell suspension containing 18·4 mg. cells/ml., liberated on boiling 17 μg. acetylcholine/mg. cells; 1 ml. of the same suspension added to 1 ml. of tyrocidin solution (1 mg./ml.) and left at room temperature for 30 min. liberated the same amount of acetylcholine, while the control suspension left in water liberated 0·59 μg. acetylcholine/mg. cells. A similar result was obtained by the use of aerosol O.T. used in the same concentration and in the same conditions as the tyrocidin.

Ten ml. of a suspension of cells (20 mg./ml.) was shaken for 6 hr. in a mechanical shaker with glass beads in order to disrupt the cells (Curran & Evans, 1942); a similar suspension was left untreated. The control suspension before boiling gave a muscle response equivalent to 0·5 μg. acetylcholine/ml., after boiling 2·2 μg./ml. The disrupted cell suspension gave 2·42 μg. acetylcholine/ml. before boiling and 2·40 μg./ml. after boiling. Thus both surface-active agents and mechanical disruption liberated the acetylcholine from the cells.

Formation of acetylcholine in washed suspensions

The cells grown in any of the media described will, after centrifuging and washing, synthesize acetylcholine in medium 6, without further growth. It is seen from Table 4 that the non-proliferating cells continue to synthesize acetylcholine, which increases about threefold; it is also seen that, whereas at the start of the experiment 90 % of the acetylcholine was present in the cells, at the end of the period 2 % was found in the cells and 98 % in the medium. A method for studying acetylcholine formation apart from cell multiplication having been arrived at it became possible to study the process in further detail.

Table 4. *The synthesis of acetylcholine by cell suspensions of* Lactobacillus plantarum

Cells grown on a mixture of equal volumes of cucumber juice and medium 2 at 25° for 20 hr. Cell suspension (3·9 mg. dry wt. cells/ml.) 1 ml. + 9 ml. reaction medium 6 incubated at 25°. Samples withdrawn at 0 hr. and 20 hr.

	Acetylcholine			
	μg./ml.	μg./mg. dry wt. cells	μg./ml.	μg./mg. dry wt. cells
Period of incubation (hr.)	0	0	20	20
Suspension before boiling, *A*	0·3	0·77	7·5	19·2
Suspension after boiling (total), *B*	3·0	7·7	9·4	24·0
B−*A* (stored in cells)	2·7	6·93	1·9	4·8

Relation between concentration of bacterial suspension and rate of formation of acetylcholine. The cells were grown as usual, centrifuged, washed and suspended in varying concentrations in the reaction medium containing 2 % glucose; incubation was at 25°. The rate of formation of acetylcholine was established by withdrawing samples and estimating acetylcholine at 0, 2 and 4 hr. for each concentration of cells used. Fig. 3 shows that in the conditions of this experiment the acetylcholine formed was proportional to the dry weight of cells present between 0 and 0·66 mg./ml.

Temperature optimum. Fig. 4 shows that the rate of synthesis was higher at 20° than at 37°; this is not in accordance with the optimum temperature for fermentation, which is 40°. As in most cases the experimental period was 2–4 hr. and never more than 20 hr., the experiments were carried out at 25° on account of the greater ease of maintaining constancy at this temperature.

Effect of anaerobiosis. In the expectation that anaerobic conditions would favour the reaction, aerobic and anaerobic conditions were tried; the aerobic reaction was carried out in small Erlenmeyer flasks, the anaerobic reaction in evacuated Thunberg tubes. The results are shown in Fig. 5. The difference is not very marked and subsequent work was carried out aerobically.

The effect of various constituents of the reaction medium. An experiment was carried out to determine which constituents of the reaction medium were essential to the synthesis of acetylcholine. Acetate and phosphate replaced McIlvaine's buffer, and glucose and choline were in turn omitted. From Table 5 it appears that in the absence of choline or of glucose no synthesis occurred, and that acetate buffer could replace citrate + phosphate mixture; also that added phosphate was not essential.

An analogous increase in the formation of acetylcholine due to the presence of glucose has been noted in the case of brain slices (Quastel, Tennenbaum & Wheatley, 1936); in this case the action was aerobic.

Concentration of glucose and age of culture. Fig. 6 shows that the rate of synthesis of acetylcholine was the same in 0·1 M- and 0·02 M-glucose, but that in

0·01 M-glucose there was a significant drop. The age of the culture was not of great importance in the synthesis (Fig. 7).

Correlation between acetylcholine synthesized, glucose fermented and change in pH. Fig. 8 and Table 6 show that synthesis proceeded smoothly so long as any glucose remained and then ceased, also that the ratio of acetylcholine synthesized to glucose fermented remained remarkably constant over the period of the experiment. Pyruvate could not replace glucose.

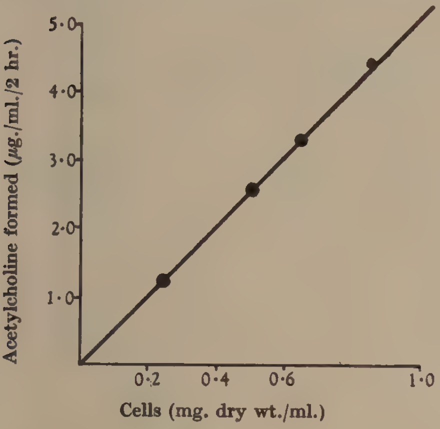

Fig. 3

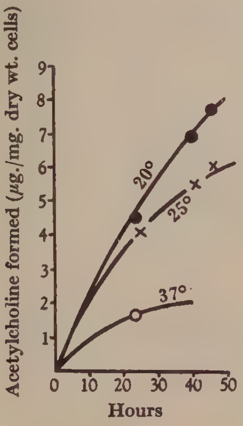

Fig. 4

Fig. 3. Effect of concentration of cells on rate of formation of acetylcholine.

Fig. 4. Effect of temperature on rate of formation of acetylcholine.

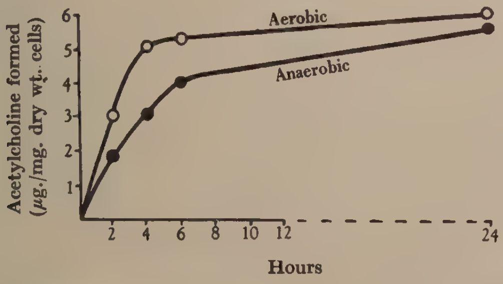

Fig. 5. Effect of anaerobiosis on rate of formation of acetylcholine.

The action of some inhibitors on the fermentation and on acetylcholine production is indicated in Table 7, and the action of certain phosphorylated derivatives on the synthesis of acetylcholine in Table 8.

Effect of the absence of choline from the growth medium

Medium 4 was devised to show whether the organism could grow without the production of acetylcholine. Cultures grown on medium 4 produced no measurable amount of acetylcholine in the cells or culture fluid. For example, 134 µg. acetylcholine/mg. cells was produced in the presence of choline and <0·17 µg./mg. cells in its absence, the growth amounting to 0·091 and 0·092 mg./ml. in the two cases. When the mixture of amino-acids was replaced by an acid hydrolysate of casein as in medium 5, the production of acetylcholine was either very low or nil, depending apparently on the sample of casein used in making the hydrolysate. When choline was added to this

Table 5. *The effect of various constituents of the culture medium on the formation of acetylcholine by* Lactobacillus plantarum

The cell suspension of the organism (grown as in Table 4) contained 10·8 mg. dry wt. cells/ml. Reaction mixtures as indicated below were made up with water to a final volume of 5 ml., and incubated at 25° for 20 hr.

Medium constituent	Composition of reaction mixtures (ml. of appropriate solutions)				
Sodium acetate, M	0·5	0·5	0·5	0·5	0·5
KH_2PO_4, M/15	0·2	0·2	0·2	0	0·2
$MnSO_4+MgSO_4$, 0·2 %	0·5	0·5	0·5	0·5	0·5
Cysteine, 0·02 g./ml.	0·5	0·5	0·5	0·5	0·5
Glucose, 2 %	0·5	0·5	0	0·5	0·5
Choline HCl, 0·5 %	0·5	0	0·5	0·5	0·5
Cell suspension	0·5	0·5	0·5	0·5	0
	Acetylcholine µg./ml.				
Acetylcholine found in excess of that initially present in organism	20	0	0	24	0

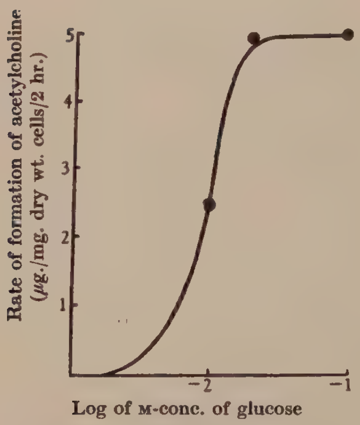

Fig. 6

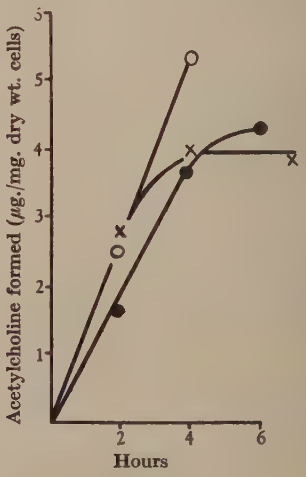

Fig. 7

Fig. 6. Effect of glucose concentration on rate of formation of acetylcholine.

Fig. 7. Effect of age of culture on rate of formation of acetylcholine. Age of culture ●—● 16 hr.; ×—× 20 hr.; ○—○ 24 hr.

medium in the usual concentration acetylcholine appeared, but the crop of cells was the same. When the cells in each case were centrifuged, washed and added to the reaction medium, acetylcholine was synthesized at approximately the same rate in both cases (Fig. 9).

Table 6. *The correlation between acetylcholine synthesized, glucose fermented and change in* pH

Organism grown on medium 5. Reaction medium; phosphate citrate buffer 10 ml.; $MnSO_4$ (4 %) + $MgSO_4$ (4 %) 5 ml.; choline HCl (0·5 %) 5 ml.; glucose (0·1 M) 10 ml.; cell suspension (6 mg. dry wt./ml.) 10 ml.; water to 50 ml. Incubation at 25°. Samples removed at times stated.

Period of incubation (hr.)	pH	Glucose		Acetylcholine formed	
		Present (mg./ml.)	Used (mg./ml.)	µg./ml.	µg./mg. glucose fermented
0	7·14	3·45	0·00	0·0	—
1	6·6	2·19	1·26	2·0	1·59
2	6·08	1·39	2·06	4·6	2·23
3·3	5·55	0·57	2·88	6·6	2·29
5·3	4·83	0·043	3·41	8·0	2·35
24	4·73	0·01	3·44	8·0	2·33

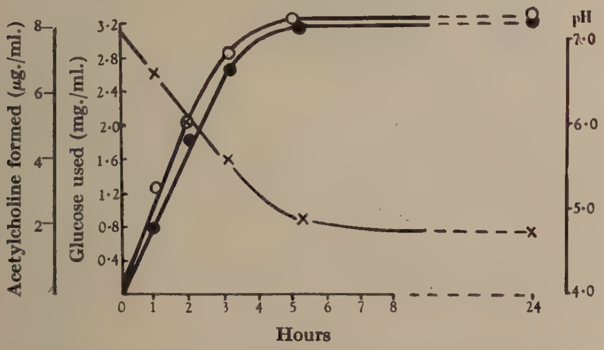

Fig. 8. Correlation between acetylcholine synthesized, glucose fermented and change in pH. ●—●, glucose fermented; ○—○, acetylcholine formed; ×—×, pH.

The proven origin of acetylcholine from choline in the reaction medium makes it highly probable that it has a like origin in cucumber juice and in ordinary complex laboratory media. Choline has been isolated from cabbage by Keil & Gropp (1934) but was stated to be absent from cucumber juice by Keil & Kritter (1935).

It was found possible to acetylate choline by the method of Fletcher, Best & Solandt (1935). This acetylation was only claimed by the authors to be very roughly quantitative (± at least 15 %). Its application to cucumber juice and to acid and tryptic digests of casein made it clear that these materials contained

Table 7. *The action of various inhibitors on fermentation and acetylcholine production*

	Inhibition (%) on	
	Fermentation	Acetylcholine production
Iodoacetic acid M/250	96	100
Na fluoride M/50	Increased lag	20
Phloridzin M/20	—	0
Na arsenite M/250	—	90
Na azide M/1000	—	0

Table 8. *The action of phosphorylated compounds on the synthesis of acetylcholine*

Experimental conditions as in Table 6, the substances shown being incorporated in the reaction medium. Time of incubation 3 hr.

	Acetylcholine (μg./ml.)
Glucose, 0·5 %	7·2
Glucose-1-phosphate, 1 %	6·0
Hexose diphosphate, 1 %	1·2
Na glycerol phosphate, 0·5 %	1·7
Na phosphoglycerate, 0·5 %	0·6 (neg.)
Blank	0·29

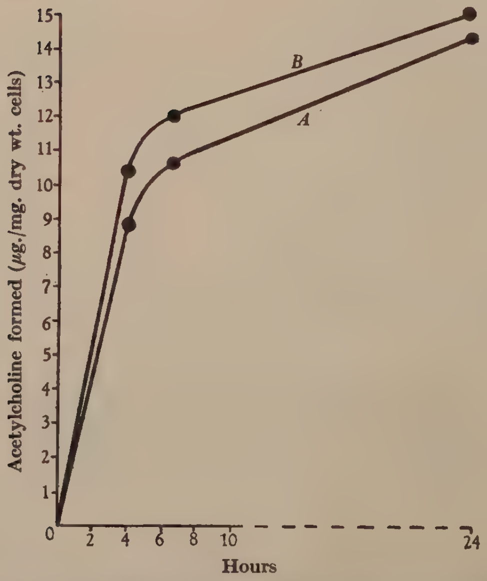

Fig. 9. Synthesis of acetylcholine by cells grown: *A*, without choline; *B*, with choline.

choline in sufficient quantity to account for the production of acetylcholine by the action of the organism in the presence of glucose (Table 9).

Table 9. *The acetylation of choline in various media by the method of Fletcher, Best & Solandt* (1935)

Choline HCl equivalent to 40 μg. acetylcholine in 5 ml. water was treated and acetylated according to the method of Fletcher *et al.* (1935), with the following results:

Choline taken (μg.)	Choline equivalent of acetylcholine obtained μg.	% error
40	39	− 2·5
40	47	+ 17·3
40	45	+ 12·5
50	54·5	+ 9
50	43	− 14

In a similar manner estimations of the choline content of cucumber juice and tryptic and acid hydrolysates of casein (10 %) were made:

	Choline (as acetylcholine) (μg./ml.)
Cucumber juice	75–80
Tryptic hydrolysate of casein (10 %)	40
Acid hydrolysate of casein (10 %)	20

Quantitative relation between choline present and acetylcholine formed

It will be noticed that in the reaction medium choline HCl was present in a final concentration of about 500 μg./ml.; the acetylcholine formed in the most favourable circumstances was of the order of 16 μg./ml. It should therefore be possible to diminish the concentration of choline added to one more nearly equivalent to the acetylcholine produced. An experiment of this type showed that a decrease in the concentration of choline from 400 to 16 μg./ml. diminished the rate of the reaction by about one-sixth. In order, therefore, for the reaction to proceed at something approaching maximum velocity a large excess of choline is necessary. It was thought that this might be due to the removal of choline by some reaction other than by acetylation. To test this the organism was grown for 40 hr. in medium 5 containing approximately 2·1 μg. choline/ml. as estimated after chemical acetylation; to this was added 40 μg. choline HCl/ml.; a control sample was acetylated and the acetylcholine estimated. The total initial choline was equivalent to 50 μg. acetylcholine/ml. After 40 hr. incubation the acetylcholine was 4·6 μg./ml. The whole culture was then acetylated, and the resulting total acetylcholine obtained was 53 μg./ml. This experiment showed that the choline originally present was recoverable as acetylcholine formed biologically *plus* that remaining in the medium.

We have not found conditions in which the rate of acetylcholine formation is proportional to the concentration of choline present, or independent of the concentration, or in which the whole of the choline present is acetylated.

Attempts to prepare active acetone-dried cells

It has been observed in studies on the production of acetylcholine by nervous tissue that homogenized brain synthesized acetylcholine in the presence of adenosine triphosphate (ATP) and fluoride and absence of carbohydrate or any source of energy other than ATP (Nachmansohn & Machado, 1943). Feldberg & Mann (1945–6*a*) prepared an active acetone powder from brain which, in the presence of ATP and NaF, acetylated choline, the rate of the reaction being increased by —SH compounds and by citrate. Feldberg & Mann (1945–6*b*) also showed that the rate of the reaction was increased by the addition of an 'activator', i.e. boiled juice prepared from acetone powder from brain or from fresh brain, liver, muscle or yeast; they were also able to replace the acetone powder by a saline extract of it.

It was considered desirable to show whether a similar preparation, active in the presence of ATP, could be prepared from our bacterial cells. The organism was grown for 2 days in 3300 ml. of medium 5; this medium was chosen in order to minimize the acetylcholine present in the cells. The total crop was 2·8 g. dry wt. This was spun down and washed once and shown to be active in the normal conditions. The cells were suspended in 15 ml. water and rubbed up with 30 ml. of cold acetone, filtered on a Buchner funnel, again rubbed up with acetone, filtered and dried in a vacuum desiccator; the yield of dry powder was 2·79 g. This powder, in amounts about 10 times the equivalent of fresh cells generally employed, was used in the experiment recorded in Table 10, kindly performed for us by Dr Feldberg in conditions identical with those used by him for acetone powder of brain. It is seen from the results (Table 10) that there was no evidence of any appreciable activity of acetone preparations of bacterial cells in the presence of ATP, with or without glucose or activator.

Thus we have so far obtained no information concerning the mechanism of the transfer of the energy derived from the bacterial fermentation to the acetylation of the choline.

The effect of pantothenic acid

In December 1946 at Cambridge Dr F. Lipmann of the Massachusetts General Hospital, U.S.A., gave an informal lecture on acetylations by liver tissue in which he had found the participation of a coenzyme involving pantothenic acid. Entirely as a result of this information (Lipmann, Kaplan, Novelli, Tuttle & Guirard, 1947) we performed the following experiment:

The cells were grown on medium 5 from which pantothenate was omitted. The washed suspension was divided into two portions, *A* and *B*. *A* was incubated in reaction medium 6, *B* in the same medium in which was incorporated calcium pantothenate, 10 μg./ml. The final concentration of the bacterial suspension was 0·96 μg./ml. Samples were withdrawn at intervals and the acetylcholine estimated. The results are shown in Fig. 10. The glucose used in 6 hr. was 875 μg./mg. dry wt. cells in *A* and 948 μg./mg. dry wt. cells in *B*. Our experiment should be regarded only as confirming Dr Lipmann's observations.

Table 10. *Inability of an acetone-dried preparation of cells of* Lactobacillus plantarum *to form acetylcholine under conditions which would have permitted its formation by homogenized rat brain*

Cells obtained from 2-day growth in medium 5, centrifuged off, washed and dried with acetone and used to set up the experiments detailed below.

	Composition of experimental mixtures							
	1	2	3	4	5	6	7	8
Phosphate buffer pH 7·2 ml.	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5
Saline (ml.)	2·0	2·4	2·0	0·4	—	2·0	—	—
KCl (mg.)	6	6	6	6	6	6	6	—
NaF (mg.)	3	3	3	3	3	3	3	—
Citrate (mg.)	15	15	15	15	15	15	15	—
$MgCl_2$ 4 % (ml.)	1	1	1	1	1	1	1	—
Cysteine (mg.)	0·45	0·45	0·45	0·45	0·45	0·45	0·45	0·45
Glucose (mg.)	—	—	—	—	—	—	25	25
Choline HCl (mg.)	—	2·5	2·5	2·5	2·5	2·5	2·5	—
ATP (mg. pyro-P)	0·4	—	0·4	—	0·4	0·4	0·4	—
Boiled rat brain (mg.)	—	—	—	80	80	—	80	80
Acetone powder (mg.) (from *Lb. plantarum*)	50	50	50	50	50	50	50	50
Total vol. reacting (ml.)	5	5	5	5	5	5	5	5
Amounts of acetylcholine found µg./mg. powder	1·0	1·1	1·1	1·3	1·5	1·0	1·4	1·2

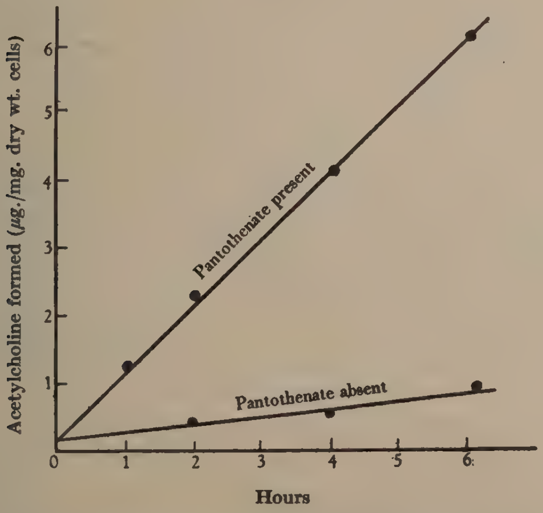

Fig. 10. **Effect** of added pantothenate on formation of acetylcholine by pantothenate-deficient cells in washed suspension.

Identification of acetylcholine

Our controls have invariably shown that the substance to which the muscle reacts is destroyed by boiling at pH 10. It was also destroyed by horse plasma and by human plasma and serum, and protected from these effects by eserine; the plasmas and serum were able to decompose added acetylcholine (Table 11).

Table 11. *The biological identification of acetylcholine; the effects of eserine and choline esterase-containing preparations*

The organism was grown on medium 2, boiled and neutralized to pH 7·0; this is the culture fluid. The experimental tubes containing the mixtures detailed below were incubated for 1 hr. at 37°, being made up to a total final volume of 2·0 ml. with water. Choline esterase: 1 = 50 % horse plasma; 2 = human plasma; 3 = human serum.

Constituent of mixture	ml. of solutions listed								
Culture fluid	1	1	0	1	0	1	1	1	0
Acetylcholine (10 μg./ml.)	0	0	1	0	1	0	0	0	1
Eserine 1 μg./ml.	0	0	0	0	0	0·2	0	0	0
Buffer pH 7·0	0	0	0	0	0·8	0	0	0	0
Choline esterase: 1	0	1	1	0·8	0	0	0	0	0·8
2	0	0	0	0·2	0·2	0·2	0	0·2	0
3	0	0	0	0	0	0	0·2	0·2	0·2
Water	1	0	0	0	0	0·6	0·8	0·6	0
Acetylcholine found (μg./ml.)	5	0	0	0	0	5	0	5	0

The biological tests being in accordance with the identification of the active substance as acetylcholine it was felt that corroboration by a chemical method was desirable. Mr K. Harrison undertook to do this, by the method detailed later (see Addendum), if sufficient material could be provided. Large-scale preparations were therefore undertaken. These were attempted in the first place by growing the organism in 1 l. batches in cucumber juice. It was found in all preparations of this volume that poor yields were obtained by using small inoculations, and that it was necessary to sow each 1 l. batch from a pilot preparation of a 20 hr. culture in 100 ml. of the same medium. From such a culture (cf. culture 1, Table 12) grown for 22 hr. a sample was withdrawn and the cells and total acetylcholine estimated. The whole culture was then centrifuged and the acetylcholine estimated in the supernatant. The cells were made up to 27 ml. with 0·01 N-H_3PO_4. A sample (0·5 ml.) was withdrawn for turbidimetric estimation and a similar sample for acetylcholine estimation after boiling in acid. In order to increase further the yield the cells were suspended in 250 ml. of reaction medium 6 and incubated 20 hr. (culture 2). A sample (1 ml.) was then withdrawn and the total acetylcholine estimated. The whole culture was then centrifuged and washed and the acetylcholine estimated in this second supernatant (2) and in the cells (2). The results of this experiment are given in Table 12.

It was hoped that in the precipitation method it would be possible to use the acetylcholine present in the total culture on cucumber juice after the removal of the cells. This was found to be impossible, as when the precipitating agent—hexanitrodiphenylamine—was added to the concentrated filtrate a tarry precipitate was formed. For this reason the second process detailed above was resorted to, in which the supernatant from the growth medium was discarded and the cells resuspended in the reaction medium. Here again trouble was encountered owing to the presence of residual choline which also forms a salt with the reagent. Though the solubility of the choline salt is about 10 times that of the acetylcholine salt, the amount of residual choline may be 100 times that of the acetylcholine and the separation of the two would therefore be practically impossible.

Table 12. *Method of preparation of large batches of cells and culture fluid for chemical identification of acetylcholine*

Culture 1. Cucumber juice (1000 ml.); inoculated with 100 ml. of 20 hr. culture grown on same medium, and incubated 22 hr. at 25°; acetylcholine estimated in sample of whole culture, cells and supernatant:

	mg.
Dry wt. of cells	490
Acetylcholine in total culture	28
Acetylcholine in total cells	6·48
Acetylcholine in total supernatant	15·4
Loss	6·1

Culture 2. Cells from culture 1 (less samples) suspended in 250 ml. of medium 6 and incubated for 20 hr. at 25°, similar assays performed.

	mg.
Acetylcholine in cells (start)	6·2
Total acetylcholine in culture after incubation	23·0
Acetylcholine in supernatant after incubation	8·8
Acetylcholine in cells after incubation (by difference)	14·2

The only feasible method therefore seemed to be to discard both supernatants 1 and 2 and to use the acetylcholine from the cells after reacting in medium 6. The following slightly modified procedure was therefore adopted. Medium 2 (1 l.) was sown with 100 ml. of a 20 hr. culture in the same medium. After 20 hr. incubation at 25° the culture was centrifuged and the supernatant discarded. The cells (*c.* 200 mg.) were resuspended in 50 ml. reaction medium 6 and left 4 hr. The cells were then spun off and the second supernatant discarded. The cells were suspended in 10 ml. of water + 1 ml. $2\,\text{M-}H_3PO_4$, boiled, and the acetylcholine estimated; the cell debris was centrifuged off and the supernatant used for the subsequent precipitation. In this manner 102 l. of culture were worked up in nine batches. This gave a total crop of 23 g. of cells and 111 mg. of acetylcholine.

DISCUSSION

The bacterial acetylation of choline bears some relation to that occurring in other tissues, viz. that the source of energy is fermentation; the organism being practically an anaerobe, the replacement of fermentation energy by that derived from oxidation is not to be expected. The mechanism of the transfer and the source of the acetyl radicle are at present obscure. Our failure to demonstrate the participation of ATP in the process is probably due to a failure to attain the right conditions rather than to the existence of some mechanism of energy transfer in which this ubiquitous ester plays no part.

The significance of the mechanism in the cell economy is wholly unknown. It has to be remembered that this is an example of a cell mechanism which would not have been brought to light unless a very delicate method existed for the detection and measurement of the final product. The same mechanism may be used to bring about other acetylations in the cell about which we know nothing. The lack of correspondence between the concentration of choline required to permit maximal—or near maximal—acetylation and the amount acetylated indicates that there is some factor or factors remaining to be disclosed.

Addendum

The Isolation of Acetylcholine as a Salt (Hexylate) of Hexanitrodiphenylamine

By K. HARRISON

Ackermann & Mauer (1943) proposed the use of hexanitrodiphenylamine (dipicrylamine) as a precipitant for acetylcholine; the salt (or 'hexylate') with acetylcholine forms beautiful glistening red plates, m.p. 125° (corr.), soluble in water to the extent of 1 : 10,000 only. (It would seem that the uncorrected m.p. 183°, given by Ackermann & Mauer, is a typographical error.) Choline hexylate, m.p. 233° (corr.), is much more soluble in water.

To prepare the hexylate of acetylcholine the procedure was as follows: A warm filtered aqueous solution of the ammonium salt of hexanitrodiphenylamine ('Aurantia') was added to the neutralized extract obtained from the boiled suspension of cells previously described; after bringing the mixture to 60° and cooling in ice the hexylate was filtered off and recrystallized, first from 10% (v/v) ethanol in water and then from water. Authentic specimens of acetylcholine hexylate were obtained in similar fashion from neutralized acetylcholine chloride (Roche Products Ltd. and British Drug Houses Ltd.).

Owing to the high pharmacological activity of propionylcholine (Chang & Gaddum, 1933), it was deemed advisable to prepare propionylcholine hexylate also. Propionylcholine chloride was synthesized by the method of Cline (1934); analytical figures (Dr Weiler and Dr Strauss) and melting-points are summarized in Table 13. It will be observed that these hexylates (in common with other aromatic nitro-compounds) tend to give high figures for C and low figures for N, due to traces of NO_2 being estimated as CO_2; if the difference, C found $-$C calc., be transferred to N found, the analytical figures are very satisfactory.

Taking the evidence of melting-points together with the analytical results there seems to be no doubt that the substance produced by the organism used in this work is acetylcholine, thus confirming the work of Keil & Kritter (1934). The recovery of acetylcholine in the form of hexylate from the neutralized bacterial extract amounted to about 75 % of the acetylcholine determined biologically. The high yield of hexylate provides further, if indirect, evidence that the bulk of the active substance is acetylcholine; although the possibility that traces of propionylcholine may also be present cannot be excluded, it seems to be remote.

Table 13. *Melting-points and analytical figures for acetylcholine hexylate and propionylcholine hexylate*

	m.p. (corr.)
1. Acetylcholine hexylate (from acetylcholine chloride)	125°
2. Acetylcholine hexylate (from acetylcholine prepared from cells)	124°
3. Mixture of 1 and 2	124°
4. Propionylcholine hexylate (from propionylcholine chloride)	113°
5. Mixture of 2 and 4	Below 108° (indistinct)

Analytical results

		C	H	N %
1.	Calc.	39·03	3·45	19·19
	Found	39·3	3·35	18·8
2.	Found	39·6	3·53	18·5
4.	Calc.	40·1	3·71	18·74
	Found	40·7	3·76	18·1

The authors wish to record their thanks to Mr H. Mowl for help in the biological estimation of acetylcholine.

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The Assimilation of Amino-acids by Bacteria

4. The Action of Triphenylmethane Dyes on Glutamic Acid Assimilation

By E. F. GALE and P. D. MITCHELL

Medical Research Council Unit for Chemical Microbiology,
Biochemical Laboratory, Cambridge

SUMMARY: The level of glutamic acid concentration measured inside the streptococcal cell during assimilation represents a balance between the rate at which the amino-acid is withdrawn from the external medium and the rate at which it is metabolized within the cell.

Treatment of the cells, prior to or during assimilation, with dyes of the triphenylmethane series results in raising the level of free glutamic acid attained within the cell. Evidence is presented that the triphenylmethane dyes prevent the metabolism of glutamic acid within the cell. The activity of the triphenylmethane molecule as an antibacterial agent and in raising the level of glutamic acid assimilation is increased by alkyl substitution and can be correlated with the lipid solubility of the dye.

Dyes of the triphenylmethane series have long been known as effective antibacterial agents. Kligler (1918) showed that the quinonoid structure of the dye molecule is important for antimicrobial activity and that the effectiveness of the dyes is increased by substitution of alkyl radicles in the amino-groups of triamino- and diamino-triphenylmethanes. He showed that bacteria differ widely in their sensitivity to these dyestuffs and that, in general, Gram-positive organisms are more sensitive than Gram-negative species. The dyes fell into the following order of increasing effectiveness: fuchsin ($1\,CH_3$), methyl violet ($5\,CH_3$), crystal violet ($6\,CH_3$), malachite green ($4\,CH_3$), brilliant green ($4\,C_2H_5$). A series of studies by Stearn (1927) and Stearn & Stearn (1924, 1925, 1926, 1930) led these workers to put forward a theory of dye bacteriostasis which can be summarized as follows: Basic dyes combine reversibly with acidic groups of bacterial protein, and the action of a dye can be described in terms of a mass-action equilibrium between the dye molecule and the organism considered as an ampholyte. They showed that Gram-positive organisms have an isoelectric range lying between pH 2 and 3, while the isoelectric range of Gram-negative organisms averages pH 5; also that the degree of Gram-positiveness of bacterial cells parallels their sensitivity to triphenylmethane dyes and that the loss of Gram-positiveness (by mutation) is accompanied by a shift in the isoelectric range of the organism towards the range of Gram-negative organisms. McCalla (1941, 1942) and McCalla & Clark (1941) showed that dyes are taken up by bacteria by ionic interchange, basic dyes displacing cations and acidic dyes, anions; thus crystal violet displaces magnesium or hydrogen ions. Although, as suggested by Stearn & Stearn (1924), the basicity of the dye is one of the main factors determining the bacteriostatic effectiveness, these workers and Kligler (1918) realized that this is not the only factor, as methyl green (heptamethyltriaminotriphenylmethane) with a strongly basic quaternary-N group, is without antibacterial activity. Studies on other types of cell have suggested that the plasma membrane is a protein-lipid complex (Harvey & Danielli, 1938; Schulman, 1937), and that a further factor involved in the increasing activity of the dyes with alkyl substitution is the increasing lipid-solubility with such substitution in the molecule. Marshall, Lockwood & Dubos (1941) pointed out that Gram-negative bacteria have an outer surface of lipopolysaccharide antigens.

In the previous papers of this series it has been shown that Gram-positive bacteria differ from Gram-negative in that the former are able to assimilate certain amino-acids from the medium and concentrate them in the free state in the internal environment (Gale, 1947*a*; Taylor, 1947). Of the two amino-acids studied in detail, lysine passes across the cell wall by simple diffusion under an electrostatic gradient, while glutamic acid cannot pass across the membrane unless energy is supplied by some exergonic reaction. In either case an equilibrium is set up between the external and internal concentrations of the amino-acid such that the internal concentration is markedly greater than that holding in the external environment (Gale, 1947*a*). It has been suggested that this assimilation of free amino-acids is an essential stage in the anabolism of the Gram-positive organisms which are nutritionally exacting towards certain amino-acids (Gale, 1947*a*). In the present communication it is shown that the free glutamic acid measured in the internal environment of Gram-positive cells represents a balance between the rate of assimilation of glutamic acid from the external environment and the rate at which it is metabolized within the cell. Triphenylmethane dyes penetrate the cell wall and inhibit the internal metabolism of glutamic acid.

Methods

The organisms, growth media, preparation of suspensions and methods of assay of free amino-acids in the internal and external environments were the same as those described by Gale (1947*a*). In general, *Streptococcus faecalis* was grown in a medium containing little free glutamic acid; the cells were washed and their internal glutamic acid content assayed; the cells were then suspended in a salt solution containing a known amount of glutamic acid and glucose and incubated at 37° for 1 hr. (final suspension strength = 2–5 mg. dry weight of organism/ml.); the cells were then centrifuged, washed once and their internal glutamic acid content assayed.

Since glutamic acid was estimated manometrically in terms of CO_2 produced by the action of the specific decarboxylase (Gale, 1947*a*), it has been convenient throughout to express quantities of glutamic acid in terms of μl.; 22·4 μl. glutamic acid = 1 μmol.

Effect of crystal violet on glutamic acid assimilation

Preliminary work. The action of various antibacterial substances has been tested on the assimilation of lysine and glutamic acid by washed suspensions of *Strep. faecalis* ST. In the course of these tests it was found that gentian violet or crystal violet in a dilution of 1 : 1000 inhibited the uptake of glutamic acid by deficient cells, but the same dyes in 1 : 10,000 solution gave rise to an increased assimilation. Table 1 shows μl. glutamic acid assimilated by 100 mg. deficient cells when incubated for 1 hr. at 37° in the presence of salts, 0·5 % (w/v) glucose and 200 μl. glutamic acid/ml. The amount of glutamic acid assimilated was determined by measuring the increase in the internal glutamic acid content of the cells under these conditions. When glucose is omitted from the external environment, no significant assimilation occurs whether crystal violet is present or not; the addition of crystal violet (1:10,000) in the presence of glucose resulted in an increase of 148 μl. of glutamic acid assimilated by 100 mg. of cells. Crystal violet forms micelles in solution, and it was thought possible that the extra glutamic acid might be carried into the cells in solution in the dye micelles. To test this the cells were exposed to 1 : 10,000 crystal violet for 10 min. at 37° and then washed before being incubated in the presence of glutamic acid and glucose. Table 1 shows that such pretreated cells showed the same increase as those in which the dye was present during the assimilation process. The action of the dye is therefore to affect the cells in such a way that within the stained cells a greater accumulation of free glutamic acid takes place than in the untreated cells.

Table 1. *Effect of crystal violet on glutamic acid assimilation*

Strep. faecalis grown in deficient medium B, cells washed and their internal glutamic acid content assayed, cells then incubated for 1 hr. at 37° as below and the increase in internal glutamic acid determined.

External environment during incubation	μl. glutamic acid increase in internal environment/100 mg. cells
(*a*) Salt solution pH 7·4; 0·5 % (w/v) glucose; glutamic acid 200 μl./ml.	113
(*b*) As (*a*) without glucose	9
(*c*) As (*a*) + crystal violet 1:10,000	261
(*d*) As (*c*) without glucose	3
Cells treated with crystal violet (1:10,000) for 10 min., washed and then tested:	
(*e*) As (*a*)	239
(*f*) As (*b*)	5

Effect of dye concentration. Table 2 shows the effect of various concentrations of crystal violet on the assimilation of glutamic acid and also on the fermentation of glucose by the washed streptococcal cells. Crystal violet at 1:1,000,000 had no effect on either assimilation or fermentation; higher concentrations resulted in an increased accumulation of glutamic acid in the internal environment of the cells. The optimal effect on assimilation was given with 1:10,000 crystal violet whereas 1:1000 dye inhibited assimilation. These concentrations of dye had an inhibitory action on fermentation when this was determined manometrically by measuring the formation of lactic acid in bicarbonate/CO_2 buffer. Dye (1:10,000) inhibited acid production by approx. 45 %; 1:1000 dye produced 96 % inhibition and this concentration of crystal violet was bactericidal.

The concentration of dye which is bacteriostatic for this organism was determined by taking tubes of casein digest glucose media, adding serial dilutions of crystal violet, inoculating with a standard inoculum of *c.* 10^6 cells/ml. and incubating at 37° for 24 hr. A dye concentration of 1:500,000 prevented growth while 1:700,000 allowed normal growth. No significant alteration of this bacteriostatic concentration could be obtained by varying the amino-acid composition or concentration of the test medium. Stearn (1933) showed that the bacteriostatic concentration of dye was proportional to the bacterial mass present. The dry weight of cells/ml. in the assimilation and fermentation experiments quoted in Table 2 was 1–2 mg., which is approximately 100 times the bacterial mass present in the growth tests. Consequently the dye concentration (1:10,000) giving optimal glutamic acid assimilation bears the same relation to the bacterial mass in the assimilation experiments as the concentration (1:10^6) found to be bacteriostatic in the growth inhibition tests. The amount of dye removed from solution by the cells can be estimated photoelectrically, and in a series of tests it was found that 33 μg. crystal violet/mg. dry weight of cells were removed from 1:10^5 dye solution when the cell-suspension strength was 2·5 mg./ml., while 28 μg./mg. were taken up when both dye and bacterial suspension were diluted ten times; this confirms the finding of Stearn (1933) that the amount of dye taken up was proportional to the bacterial mass and was approximately constant when the ratio of dye/bacterial mass was constant.

Table 2. *Effect of crystal violet on glutamic acid assimilation and glucose fermentation*

Strep. faecalis grown in deficient medium B; cells washed and their internal glutamic acid content assayed; cells then incubated for 1 hr. at 37° in glutamic acid (200 μl./ml.), glucose (0·5 % w/v) and crystal violet as below; the increase in internal glutamic acid determined and hence the effect of crystal violet on its assimilation. Fermentation rates determined in Warburg manometers containing 1·0 ml. suspension of cells (dry wt. 3 mg./ml.), 1·5 ml. M/80 $NaHCO_3$ and 0·5 ml. 1 % (w/v) glucose, and filled with 5 % CO_2 + 95 % N_2 gas mixture.

Concentration of crystal violet	Effect on glutamic acid assimilation (as % control without dye)	Inhibition of glucose fermentation (%)
0	100	0
1:1,000,000	100	0
1:100,000	168	4
1:50,000	509	20
1:10,000	581	46
1:2,000	234	—
1:1,000	30	96

Course of glutamic acid assimilation in presence of crystal violet. Fig. 1 shows the rate of appearance of glutamic acid in the internal environment of 20 mg. dry weight of deficient cells when these were suspended in salt solution containing glucose (0·5 % w/v) and glutamic acid (200 μl./ml.) at pH 7·2 and 37°. Glutamic acid was taken up by the cells until the new equilibrium level was reached after 15 min. When crystal violet (1:10^4) was present during the assimilation (or when the cells had been stained in such a solution before assimilation began) the rate of assimilation was slower, but the final level reached was higher than that attained in the absence of the dye. Since the assimilation of glutamic acid, in this case, is dependent upon energy obtained from glucose fermentation, the slower rate of assimilation was probably due to the inhibition of fermentation rate produced by the dye (Table 2). The difference between the final levels reached in the presence and absence of dye was variable and depended upon the age of culture of the deficient cells.

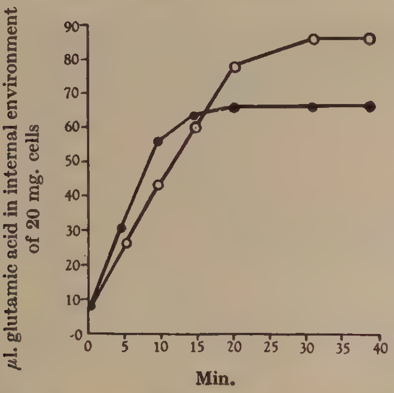

Fig. 1. **Effect of crystal violet (1:10,000) on assimilation of glutamic acid into internal environment of *S. faecalis* cells. Cells incubated at 37° in (1) salt solution + 0·5% glucose + glutamic acid (200 μl./ml.) ●—●, (2) as (1) + crystal violet 1:10,000 ○—○.**

Effect of the age of the culture. During the early stages of this work it was found that the 'dye effect' varied greatly from time to time, the same concentration of crystal violet increasing the glutamic acid assimilation from 25 to 600 % when tested under what appeared to be standard conditions. It seemed that the effect varied with the age of the cells tested, so this point was investigated in detail. Table 3 shows the μl. glutamic acid assimilated/100 mg. cells in the presence and absence of crystal violet (1:10^4) when the cells had been harvested from deficient medium at 4–17 hr. after inoculation at 37°. From the final column of Table 3 it can be seen that the assimilation in the presence of the dye was approximately constant at 450–550 μl./100 mg. from the earliest culture tested up to the tenth hour of incubation when growth has ceased; after this time the assimilation dropped rapidly. The assimilation in the absence of dye was much less from 4–10 hr. of incubation, and then that value also fell, but the two values approximated until at 17 hr. the dye had little or no enhancing effect. Cells taken at the third hour of growth also showed no dye effect, but in this case the assimilation value in the absence of dye was abnormally high and the same as that obtained in the presence of dye for all cultures up to the end of the growth period. The probable significance of these values will be discussed below. From a practical point of view it follows from Table 3 that large 'dye effects' on glutamic acid assimilation can be obtained only with cells harvested after 6–10 hr. incubation, and that older cultures will display smaller or even negligible effects of this nature.

The estimations involved in this experiment are set out in detail in Table 3 in order that another property of the 'dye effect' can be shown. The internal environment of the cells was investigated by adding glutamic acid decarboxylase to suspensions of the cells before and after disruption (Gale, 1947*a*). The glutamic acid obtained with intact cells represents that amount of the amino-acid carried down with and adsorbed on the surface of the intact cells; the value obtained with disrupted cells represents the total free glutamic acid content of the cell preparation, and the amount concentrated in the internal environment can be obtained by difference. It was noticed in the early studies of the 'dye effect' that the presence of the dye not only increased the amount of glutamic acid assimilated inside the cell but also increased the amount of glutamic acid carried down on the outside of the washed intact cells. This can be seen in Table 3: for example, cells harvested after 10 hr. growth, washed and treated by the assimilation procedure as usual gave a value of 30 μl. glutamic acid/33 mg. cell preparation for the external environment, but similar cells put through the same procedure in the presence of crystal violet gave a value of 105 μl./33 mg. It can be seen from the values for this culture in Table 3 that, while the dye caused an increased assimilation of 138 μl. in the internal environment, it also caused an increase of 85 μl. in the amount carried down outside the cells. The external effect is not always as marked as this, and it can be seen from Table 3 that the external effect is smaller in cultures both older and younger than 10 hr. This glutamic acid estimated in intact cell suspensions must exist on the outside of those cells, as the glutamic acid

Table 3. *Variation of effect of crystal violet on glutamic acid assimilation with age of culture*

Cells grown in deficient medium, washed and incubated for 1 hr. at 37° in the presence of (*a*) 0·5 % (w/v) glucose + 200 μl. glutamic acid/ml., (*b*) as (*a*) + crystal violet 1:10,000. In each case glutamic acid assayed on intact cells (external) and on cells disrupted by boiling (total); internal glutamic acid = total − external.

			Glutamic acid determinations						Internal glutamic acid (μl./100 mg. cells)	
			(*a*) No dye			(*b*) Crystal violet				
Age of culture (hr.)	Growth (mg./ml.)	Sample dry weight (mg.)	Ext. (μl.)	Total (μl.)	Int. (μl.)	Ext. (μl.)	Total (μl.)	Int. (μl.)	(*a*) Alone	(*b*) Dye present
3	0·050	6·8	31	68	37	—	—	—	545	—
4	0·137	21·6	2	58	56	67	166	99	260	460
6	0·294	34·0	24	116	92	34	212	178	286	534
8	0·310	30·0	0	84	84	50	188	138	280	460
10	0·330	33·0	20	66	46	105	289	184	138	552
12	0·330	33·3	15	52	37	65	172	107	110	320
17	—	31·6	38	72	34	34	75	41	102	123

decarboxylase preparation used for its determination is a washed suspension of *Clostridium welchii* (Gale, 1945) and obviously cannot penetrate the streptococcal cell wall. Because of this and since the amount assayed with the intact cells remained after washing the cells, it follows that this glutamic acid must be adsorbed on the surface of the cells and that the action of crystal violet was to increase the amount of amino-acid so adsorbed. It was shown in Table 1 that no increased assimilation of glutamic acid took place in the presence of crystal violet when glucose was absent; this increased adsorption of glutamic acid on the outside of the cells was also dependent upon the presence of glucose in these experiments.

Table 4. *A comparison of certain triphenylmethane dyes with respect to their inhibition of the growth of* Streptococcus faecalis *and its assimilation of glutamic acid*

(+ = Growth from inoculum of 10^6 cells/ml.)

		Conc. of dye									
Name	Substituent groups	1/10,000	1/50,000	1/100,000	1/200,000	1/300,000	1/500,000	1/700,000	1/1,000,000	Effect on glutamic acid assimilation (crystal violet effect = 100; dye concentration = 1/10,000)	Partition coefficient *iso*butanol/water
p-Rosaniline	$-NH_2$, $-NH_2$, $-NH_2$	−	−	+	+	+	+	+	+	4	12·5
Fuchsin	$-NH_2$, $-NH_2$, $-NH_2$, $-CH_3$	−	−	+	+	+	+	+	+	25	14·7
Methyl violet	$-N(CH_3)_2$ $-N(CH_3)_2$ $-NHCH_3$	−	−	−	−	−	+	+	+	85	62
Crystal violet	$-N(CH_3)_2$ $-N(CH_3)_2$ $-N(CH_3)_2$	−	−	−	−	−	−	+	+	100	76
Malachite green	$-N(CH_3)_2$ $-N(CH_3)_2$	−	−	−	−	−	−	−	+	100	125
Brilliant green	$-N(C_2H_5)_2$ $-N(C_2H_5)_2$	−	−	−	−	−	−	−	+	143	480
Methyl green	$-N(CH_3)_2$ $-N(CH_3)_2$ $-N(CH_3)_3Cl$	+	+	+	+	+	+	+	+	0	0·2
Aurine	$-OH$, $-OH$	−	−	+	+	+	+	+	+	140	625
Soluble blue	3[—NH SO_3H]	−	−	+	+	+	+	+	+	7	0·1

Activities of triphenylmethane series of dyes

Table 4 summarizes the activities of various triphenylmethane dyes when tested against *Strep. faecalis*.

Inhibiting concentration. The inhibiting concentration of each dye was determined by the addition of serial dilutions of dye to tubes containing casein digest glucose medium, inoculation with 10^6 cells/ml., and incubation for 24 hr. at 37°. Table 4 shows that the antibacterial activity of the dyes increased with the degree of alkyl substitution in the molecule. The acid dyestuffs, aurine and soluble blue, had little antibacterial activity under the conditions tested. The strongly basic methyl green with a quaternary-N group in its structure was devoid of activity.

Effect on assimilation of glutamic acid. Each of the dyestuffs was tested for an effect on glutamic acid assimilation of the nature described above for crystal violet. In each series of experiments, deficient cells were incubated for 1 hr. at 37° in the presence of 0·5 % (w/v) glucose and 200 μl. glutamic acid/ml. The assimilation of glutamic acid was determined in the absence of dye, in the presence of crystal violet (1:10,000), and in the presence of the other dyestuffs in the same concentration. All the triphenylmethane dyes, with the exception of methyl green, produced an increase in the amount of glutamic acid assimilated (as measured by the increase in the internal concentration), and Table 4 shows their relative activities when compared with crystal violet. The effects on assimilation can be directly correlated with their antibacterial activities. Alkyl substitution increased the activity of the dyes ranging from *p*-rosaniline to brilliant green, but the strongly basic methyl green was inactive. Soluble blue was almost inactive but aurine had an activity equal to that of brilliant green and so forms an exception to the general correlation occurring between assimilation effects and antibacterial activities.

Many other dyestuffs were similarly tested. The following were without effect on the assimilation process: methylene blue, toluidine blue, congo red, auramine, dispersol diazo-black, chloracol brilliant yellow, chrysaphenine, rhodamine, safranine, primulin, acridine orange, solway green, solway violet and antrypol.

Partition coefficient. If the bacterial cell membrane is of lipid nature it is possible that the difference in activity of the triphenylmethane dyes might lie in their differing lipid-solubilities. As an approximate indication of this difference the partition coefficient between *iso*butanol and water was determined.

In each case 10 mg. of dye was shaken with 10 ml. water and 10 ml. *iso*butanol saturated with water. After equilibrium was established, the alcohol and water phases were separated and the partition of the dye determined colorimetrically. The values in Table 4 show that the assimilation effects and the antibacterial activities (with the exception of aurine) can be correlated with the partition coefficient in that the greater the partition is in favour of the *iso*butanol phase, the more active is the dye in the biological systems.

The balance between assimilatory and metabolic processes

In the work so far described, the assimilation of glutamic acid has been determined by the increase in the concentration of the amino-acid in the internal environment of the cells when these are removed from an external environment deficient in glutamic acid and transferred to an environment rich in this amino-acid. It does not necessarily follow that the total amount of glutamic acid assimilated or withdrawn from the external environment is equal to this increase in the internal concentration. If, for example, some metabolism of glutamic acid takes place inside the cell, then the amount of glutamic acid withdrawn from the external environment would be equal to that appearing in the internal environment plus the amount metabolized. In this case the amount of amino-acid measured in the internal environment would in fact be a measure of the balance between the total amount withdrawn from outside and the amount metabolized within the time studied. Since the decarboxylase method estimates the free amino-acid only, such metabolism might consist of synthesis into peptide chains of glutamyl residues or of some breakdown or conversion of the glutamic acid molecule.

Effect of crystal violet on glutamic acid metabolism

The possibility that some metabolism of glutamic acid might take place during assimilation was investigated in the following manner. Deficient cells were prepared in washed suspension as usual and suspended in salt solution containing 30 μl. glutamic acid/ml. and 0·5 % (w/v) glucose. Before incubation the glutamic content of the cells and medium was assayed accurately by taking samples equivalent to the experimental samples. The cells were incubated for 1 hr. at 37° in the presence of the glutamic acid and then centrifuged, washed and their glutamic acid content reassayed. The supernatant medium and the washings were combined, evaporated *in vacuo* and assayed for glutamic acid. In this way it was possible to determine the increase in the internal concentration of glutamic acid of the cells and compare this with the disappearance of glutamic acid from the external environment, allowance being made for the alteration in the amount adsorbed on the outside of the cells before and after incubation. Table 5 outlines results obtained in a series of experiments of this nature.

Some idea of the experimental error involved in these assays and manipulations was first obtained as follows: Since glutamic acid is not assimilated by the cells in the absence of a source of energy (Gale, 1947 *a*), an experiment was first carried out in which glucose was omitted from the reaction mixture. There was no change in the internal glutamic acid concentration under these conditions but an overall loss of 64 μl. out of 1800 μl. in the external environment; this may be due to slight metabolic activity by the cells, but is probably due to loss during experimental manipulation. In the presence of glucose, a series of three experiments showed a variable increase in the amount of glutamic acid appearing inside the cells, while the amount disappearing from the external environment was greater than this increase by an average of 632 μl. In these experiments *c.* 30 % of the total glutamic acid added to the external environment disappeared during the assimilation period of 1 hr.

The same type of experiment was then carried out in the presence of crystal violet 1:10,000. In the absence of glucose a greater loss of glutamic acid was found than in the absence of the dye. Since the dye adsorbs strongly on to all glassware and so renders quantitative recovery of solutions difficult, it is probable that this increased loss was again due to experimental error and not to metabolism by the cells in the presence of the dye. In the presence of glucose the amount of glutamic acid appearing in the internal environment was markedly greater than that appearing in the absence of the dye, but the difference between the amount disappearing from the external environment and that appearing in the internal environment was now 163 µl. as compared with 682 µl. in the absence of the dye. When allowance is made for the experimental loss as measured in the absence of glucose, the glutamic acid disappearing in the absence of the dye was 568 µl. as compared with 23 µl. in the presence of the dye.

Table 5. *Effect of crystal violet on glutamic acid metabolism during assimilation*

In all cases initial external glutamic acid = 1800 µl. total; concentration of glutamic acid in external medium initially = 30 µl./ml.; total dry weight of deficient cells = 175 mg.

Present during incubation for 1 hr.				Glutamic acid determinations		Glutamic acid metabolized or lost	
Deficient cells	Glutamic (200 µl./ml.)	Glucose (0·5 % (w/v))	Crystal violet (1:10^4)	Change in internal environment (µl.)	Change in external environment (µl.)	Total (µl.)	Corrected for blank (µl.)
+	+	−	−	0	− 64	64	0
(1) +	+	+	−	+100	−712	612	
(2) +	+	+	−	+290	−882	592 } 632	568
(3) +	+	+	−	+303	−995	692	
+	+	−	+	− 5	−135	140	0
(1) +	+	+	+	+510	−785	275	
(2) +	+	+	+	+416	−555	139 } 163	23
(3) +	+	+	+	+366	−441	75	
			Aurine 1:10^4				
+	+	+	−	+320	−810	490	
+	+	+	+	+1010	−1160	150	

These experiments suggest that some metabolism of glutamic acid takes place during or after assimilation by the cells, and that this metabolism is inhibited by crystal violet. The level of glutamic acid concentration attained inside the cell thus represents a balance between the rate of entry into the cell and the rate of metabolism within the cell. Crystal violet inhibits the metabolic process and, as a consequence, the level of glutamic acid concentration within the cell rises.

A further test of this hypothesis can be obtained from a study of the metabolism of glutamic acid occurring during its migration out of the cell. It has been shown (Gale, 1947*a*) that although the cell wall will not allow free diffusion of glutamic acid, this amino-acid can pass across the cell wall in either direction if glucose is metabolized by the cell at the same time, and that an equilibrium is then established between the internal and external concentrations of glutamic acid. Consequently, if the cells are grown in a medium rich in glutamic acid, such as casein digest medium, and then incubated in a salt solution containing glucose, glutamic acid will pass out of the cell and can be detected in the external environment, although the amount found in the external medium is less than that lost from the internal environment (Gale, 1947*a*). Table 6 summarizes the results of a series of experiments of design similar to those already described, except that the cells initially contained a high concentration of glutamic acid and were incubated in an amino-acid-free salt solution. In a series of four such experiments, the net loss of glutamic acid during migration was 102 μl., but in a parallel series carried out in the presence of crystal violet, the glutamic acid accumulating in the external environment was equal to that disappearing from the internal environment within experimental error.

Table 6. *Effect of crystal violet on glutamic acid metabolism*

'Saturated cells' = cells grown in casein digest glucose and containing a high internal concentration of free glutamic acid. Total dry weight of cells = 200 mg. in all cases.

Present during incubation for 1 hr.			Glutamic acid determinations		Glutamic acid metabolized or lost	
Saturated cells	Glucose (0·5 % (w/v))	Crystal violet (1:10,000)	Change in internal environment (μl.)	Change in external environment (μl.)	Total (μl.)	Corrected (μl.)
+	−	−	− 40	0	40	0
(1) +	+	−	−335	+189	146 147	107
(2) +	+	−	−240	+ 99	141	
(3) +	+	−	−180	+ 30	150	
(4) +	+	−	−202	+ 72	130	
(1) +	+	+	−370	+335	35 26	0
(2) +	+	+	−330	+272	58	
(3) +	+	+	−102	+108	0	
(4) +	+	+	−110	+100	10	

Aurine. In Table 4 it was shown that the effect of the dyes on glutamic acid assimilation was paralleled by the partition coefficient between *iso*butanol and water, and by the antibacterial activity. Aurine, an acidic dye, differs from the others examined in that although it has a marked effect on glutamic acid assimilation, it has no marked antibacterial properties. Its action in suppressing glutamic acid metabolism has therefore been tested in parallel with the experiments described above for crystal violet. Table 5 shows the results obtained. In the presence of aurine there is suppression of glutamic acid metabolism inside the cell, but the effect is not the same as that obtained with crystal violet; examination of the figures in Table 5 shows that crystal violet inhibited glutamic acid disappearance, and that the amount of glutamic acid withdrawn from the external environment in the presence of the dye was approximately the same as, or less than, the amount withdrawn in the absence of the dye. It is reasonable to suppose that less glutamic acid will be withdrawn from the external environment when no metabolism is occurring within the cell than otherwise. In the case of aurine, however, although there was approximate balance between the loss from the external environment and the gain in the internal environment, the amount of glutamic acid withdrawn from the external environment was markedly increased by the presence of the dye. Somewhat similar results have been obtained with the acidic dye, Evans blue, which does not belong to the triphenylmethane series; in this case there was a greatly increased uptake of glutamic acid from the external medium in the presence of the dye, but there was also an increase in the amount of the glutamic acid metabolized within the cell. The dye has a feeble antibiotic action which seems to be fundamentally different from that of the triphenylmethane series.

Nature of glutamic acid metabolism

Peptide synthesis. The discovery of the structure of folic acid (Angier *et al.* 1946) and of the glutamic acid peptides associated with *p*-aminobenzoic acid (Ratner, Blanchard & Green, 1946) indicated that glutamic acid can form homoglutamic peptides in the living cell. This property is shown in the case of the D-isomer by the homo-D-glutamic acid nature of the capsular polypeptide of *B. anthracis* (Hanby & Rydon, 1946). Streptococcal protein possesses the normal complement of glutamic acid (Freeland & Gale, 1947), and it follows that one line of glutamic acid metabolism within the bacterial cell is that which leads to peptide and protein formation. If the metabolism which is blocked by crystal violet is this peptide formation, there should be an increase in combined glutamic acid during assimilation in the absence of dye, and this increase should be prevented by the presence of the dye. Combined glutamic acid of this nature would be released by acid hydrolysis and could then be estimated by the decarboxylase preparation (Gale, 1945). Table 7 shows the results of an experiment in which the free glutamic acid obtained before and after acid hydrolysis was determined with (*a*) deficient cells, (*b*) deficient cells after 1 hr. incubation with glutamic acid and glucose, and (*c*) deficient cells treated as (*b*) in the presence of crystal violet 1:10,000. The results show that there was, if anything, a decrease in the peptide-glutamic acid during assimilation. It seems, therefore, that the metabolism of glutamic acid which was blocked by crystal violet in these experiments was not peptide synthesis.

Table 7. *Absence of glutamyl-peptide formation during assimilation of glutamic acid by* Streptococcus faecalis

	μl. glutamic acid/100 mg. cells		
Preparation	Free	Total after hydrolysis	Combined (peptide)
1. Washed deficient cells	40	965	925
2. Cells after incubation for 1 hr. in 0·5 % (w/v) glucose and glutamic acid (200 μl./ml.)	225	1042	817
3. Cells treated as (2) in presence of crystal violet (1:10,000)	455	1302	847

Deamination. No ammonia formation could be detected when glutamic acid was incubated with washed suspensions of *Strep. faecalis*.

Transamination. Lichstein & Cohen (1945) and Lichstein, Gunsalus & Umbreit (1945) have shown that bacteria, including the streptococci, possess an active transaminase catalysing the formation of aspartic acid + α-keto-glutaric acid from glutamic acid + oxalacetic acid. The action of crystal violet was tested on the disappearance of glutamic acid in the presence of oxalacetic acid and a dried cell preparation of transaminase from *Strep. faecalis* kindly given to us by Prof. I. C. Gunsalus. The transamination was inhibited 75 % by crystal violet in concentration 1:100, but no significant inhibition was obtained by concentrations of the order (1:10,000) used in the experiments on assimilation.

DISCUSSION

The material presented in this paper shows that glutamic acid is assimilated by streptococcal cells from the external environment when a source of energy such as glucose is available; inside the cell some of the assimilated glutamic acid then undergoes metabolic changes so that it is no longer estimated by the specific decarboxylase preparation. The level of glutamic acid concentration estimated inside the cell then represents the balance between the rate of entry of glutamic acid into the cell and the rate at which it undergoes metabolic change within the cell. It has been shown that certain dyes of the triphenylmethane series bring about an increase in the internal concentration of glutamic acid and evidence has been put forward to show that this is accomplished by inhibition of the metabolism of glutamic acid inside the cell. The statement that the metabolism of glutamic acid takes place *inside* the cell rests upon the facts that no assimilation of glutamic takes place in the absence of glucose (Gale, 1947*a*), and no disappearance of glutamic acid from combined internal and external environments occurs in the absence of glucose. Further evidence will be presented in the next paper of this series (Gale & Taylor, 1947) that this metabolism is intracellular only. It follows that for a dyestuff to inhibit such metabolism it must first penetrate the cell wall, and this fact can probably be correlated with the variation of antibacterial efficiency of the triphenylmethane dyes and its relation, in turn, to the *iso*butanol/water partition coefficient.

The nature of the internal metabolism of glutamic acid which is inhibited by crystal violet is at present obscure. It is highly probable that some glutamic acid becomes combined into peptide chains, but this type of synthesis apparently does not occur in the washed suspension experiments described in this paper. It is shown in a later paper (Gale, 1947*b*) that this form of synthesis occurs in growing as opposed to non-growing cells. It is also probable that transamination reactions occur within the cell and involve glutamic acid; it was not possible to show inhibition of transaminase preparations by crystal violet, but this is not necessarily significant as we do not know what is the concentration or location of the dye within the intact cell. It is becoming increasingly obvious to cytologists that the organization and orientation of enzyme systems within intact cells is of primary importance for their correlated metabolic activities, and it is possible that a dye such as crystal violet may disorganize or disorientate the interior of the cell in such a way that enzyme activities may no longer be possible in ordered sequence although the dye may not be active against the cell-free enzyme systems.

It has been the custom to calculate the amount of amino-acid assimilated from the increase in the internal concentration of that amino-acid. From the results obtained in this paper it follows that such values are less than the amount of amino-acid withdrawn from the environment by that quantity metabolized during the assimilation process. A more satisfactory value can probably be calculated from the rise in internal concentration in the presence of crystal violet which prevents the internal metabolism. In Table 3 the internal glutamic acid level is given in absence (*a*) and presence (*b*) of crystal violet, and the latter series of values (*b*) presumably gives a measure of the assimilatory power of the organism at various stages during its growth. It can be seen that the μl. glutamic acid assimilated/100 mg. organism remained approximately constant until growth ceased at 10 hr. and then fell rapidly. The values in the absence of the dye were approximately constant from 4–8 hr., but began to fall off before the parallel series in the presence of dye. The value obtained with the 3 hr. culture was high in the absence of dye and of the same order as the steady figure obtained in the presence of the dye; in this case the addition of dye sometimes inhibited the glutamic acid uptake, and the culture generally behaved as though it were very sensitive to outside influences of any description. It seems possible that the high value obtained with the 3 hr. culture was actually an artefact due to inactivation of the metabolic system during preparation, this inactivation having the same effect on assimilation as the dye had in older cultures. If the ideas put forward in this paper are correct the difference between the values in columns (*a*) and (*b*) of Table 3 form a measure of the glutamic acid metabolized inside the cells of the cultures. This metabolism was greatest at 10 hr. growth—when growth was stopping—and subsequently decreased rapidly.

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The Assimilation of Amino-acids by Bacteria

5. The Action of Penicillin in Preventing the Assimilation of Glutamic Acid by *Staphylococcus aureus*

By E. F. GALE and E. SHIRLEY TAYLOR

Medical Research Council Unit for Chemical Microbiology,
Biochemical Laboratory, Cambridge

SUMMARY: The effect on glutamic acid assimilation of the addition of penicillin to growing cultures of *Staphylococcus aureus* is described. When *Staph. aureus* is grown in media containing glutamic acid this substance accumulates in steadily increasing concentration in the cells. The addition of penicillin to the medium is followed after an interval by rapidly decreasing concentration of glutamic acid within the cells.

The assimilation of glutamic acid by normal washed cells is not affected by penicillin in high concentration. The assimilation of glutamic acid by cells which have grown in the presence of penicillin is impaired and may be completely inhibited. Complete inhibition of assimilation is brought about by bactericidal concentrations of penicillin, low concentrations requiring a longer time to become completely effective than high ones. The loss of assimilatory power can be correlated with loss of viability.

Comparison of the general properties of normal and penicillin-inactivated cells show that the respiration, glucose oxidation, glucose fermentation and lysine assimilation of the latter are normal. The internal metabolism of glutamic acid is normal in penicillin-treated cells, but, since the passage of glutamic acid across the cell wall is blocked, is limited by the existing internal concentration.

Previous papers of this series (Gale, 1947*a*; Taylor, 1947) have shown that Gram-positive bacteria are able to assimilate certain amino-acids from the external environment and to concentrate these amino-acids in their internal environment so that, at equilibrium, the internal concentration is greater than the external. Lysine passes across the cell wall of *Streptococcus faecalis* by a process of diffusion, but the migration of glutamic acid requires energy which can be supplied by metabolic processes such as glucose fermentation. The maintenance of a concentration of glutamic acid within the internal environment higher than that in the external medium is dependent upon an intact cell wall (Gale & Taylor, 1947). After glutamic acid has passed through the cell wall, a portion of it undergoes metabolic change, and the level of glutamic acid concentration measured inside the cell represents the balance between the rate of entry of that amino-acid into the cell and the rate of its metabolism within the cell (Gale & Mitchell, 1947).

Since the ability to concentrate amino-acids within the internal environment is a property of Gram-positive organisms, the effect on this property of various chemotherapeutic agents has now been tested. A preliminary note on the effect of penicillin in preventing the assimilation of glutamic acid by *Staphylococcus aureus* has been published (Gale & Taylor, 1946). Penicillin is highly effective as a bactericidal agent against most Gram-positive organisms, while many Gram-negative organisms are either unaffected or affected by comparatively large concentrations (Fleming, 1929); however, the Gram-negative meningococci and gonococci are very sensitive, and the Gram-positive enterococcus, *Strep. faecalis* ST, is comparatively resistant. Penicillin does not affect the respiration of washed suspensions of susceptible staphylococci and was consequently thought to be bacteriostatic (Abraham, Chain, Fletcher, Florey, Gardner, Heatley & Jennings, 1941) but later work demonstrated that penicillin is bactericidal when it is allowed to act on cells which are growing or in a condition where multiplication is possible (Hobby, Meyer & Chaffee, 1942). Gardner (1940) found that bacteria will increase in size in the presence of penicillin but do not divide, with the result that enlarged and abnormal cells are produced. Hobby & Dawson (1944) showed that the action of penicillin can be enhanced by the presence of substances promoting bacterial growth and inhibited by substances preventing growth; this conclusion was confirmed by Chain & Duthie (1945), who showed that the bacteriostatic agent helvolic acid would protect staphylococci from the bactericidal action of penicillin, while sulphonamides, which allow several divisions to take place before inhibiting growth, had no such protective action.

Although penicillin has no action on washed suspensions of *Staph. aureus*, Hirsch (1943–4) and Chain & Duthie (1945) found that when penicillin was added to growing cultures then, after a lag period during which respiration was normal, a progressive inhibition of respiration took place which eventually resulted in complete cessation of oxygen consumption. Chain & Duthie (1945) found that when the penicillin was added in the early logarithmic phase of growth, the cells continued to grow for some time after the addition, but not more than one division per cell occurred and that abnormally large cells were produced. A loss of viability then took place which ran approximately parallel to the respiratory failure, and lysis of the non-viable cells resulted in a steady decrease in the total, as well as the viable, count. There have been few published studies of the biochemical action of penicillin, although Krampitz & Werkman (1947) have found that high concentrations (2000 Oxford units/ml.) inhibit the dissimilation of cellular ribonucleic acid and sodium ribonucleate when employed as substrate for *Staph. aureus*. Atkinson & Stanley (1943) found that the action of penicillin could be antagonized by cysteine, but further investigation seems to indicate that the penicillin molecule is inactivated by chemical reaction with a number of amino-thiol compounds (Chow & McKee, 1945; Cavillito, Bailey, Haskell, McCormick & Warner, 1945).

Methods and organisms used

Most of the present work was carried out with *Staph. aureus* strain D which was noted by Taylor (1947) to be capable of effecting a very high concentration of glutamic acid within the internal environment. The main results were checked by using another strain which effects a comparatively low internal concentration of glutamic acid and against *Strep. faecalis* ST used previously. The organisms were grown in two media: medium A being casein digest medium containing 1 % (w/v) glucose and 0·1 % (w/v) Marmite; medium B ('deficient medium') consisting of a salt mixture with 1·0 % (w/v) glucose and 0·1 % (w/v) Marmite. The preparations of penicillin used were commercial preparations (Roche Products Ltd.) of purity 500 or 800 Oxford units/mg.

The estimations of amino-acids and of assimilation were carried out as previously described (Gale, 1947*a*). In general, the organisms were grown in medium B, washed and their internal amino-acid assayed; they were then incubated in a salt solution containing glutamic acid (200 μl./ml.) and 0·5 % (w/v) glucose for 1 hr. at 37°, washed and the new internal glutamic acid level assayed.

Quantities of glutamic acid and lysine are expressed in terms of μl. (Gale & Mitchell, 1947); 22·4 μl. glutamic acid = 1 μmol.

Assimilation of lysine and glutamic acid by Staphylococcus aureus

The studies on assimilation so far described in this series were carried out with a strain of *Strep. faecalis* which is unsuitable for the investigation of the action of many chemotherapeutic agents as it is resistant to penicillin, the sulphonamides, etc. Table 1 summarizes the results of experiments carried out to determine whether *Staph. aureus* assimilates lysine and glutamic acid under conditions similar to those required by *Strep. faecalis*. The values in Table 1 show that lysine passed into the internal environment at 4°, more effectively at 37° and that the amount taken up at 37° was less when glucose was present. These results are essentially similar to those obtained in *Strep. faecalis* where it was shown that lysine passes across the cell wall by a process of diffusion, the amount assimilated being dependent upon the charge of the cell and being decreased by glycolysis. Glutamic acid did not enter the *Staph. aureus* cells at either 4 or 37° in the absence of glucose, showing that, as in the case of *Strep. faecalis*, energy was required for the migration of glutamic acid across the cell wall. In these experiments glucose has always been added as source of energy although it was shown with *Strep. faecalis* that the breakdown of arginine would also supply energy for the migration. The assimilation of glutamine by *Staph. aureus* also requires energy which can be supplied by glucolysis.

Table 1. *Assimilation of lysine and glutamic acid by* Staphylococcus aureus

Staph. aureus grown in medium B, cells centrifuged down and internal amino-acid assayed. Cells suspended in salt solution containing either 200 μl. lysine or 200 μl. glutamic acid/ml. as below, left for 1 hr., centrifuged out of suspension, washed and internal environment re-assayed. Assimilation expressed as μl. increase in amino-acid content of internal environment of 100 mg. dry weight of cells.

		Increase in internal content	
External medium	Temp.	Lysine (μl.)	Glutamic acid (μl.)
Lysine	4	30	—
Lysine	37	114	—
Lysine; glucose	37	66	—
Glutamate	4	—	Nil
Glutamate	37	—	Nil
Glutamate; glucose	37	—	402

Effect of penicillin. Table 2 shows the effect of the presence of penicillin on glutamic acid assimilation by normal cells of *Staph. aureus.* Two Oxford units/ml. had no effect on assimilation, 20–50 units/ml. gave rise to *c.* 10 % decrease in the amount of glutamic acid assimilated by 100 mg. of deficient cells under the test conditions. It is doubtful whether this decrease is significant.

Table 2. *Effect of penicillin on glutamic acid assimilation by washed normal cells*

Deficient cells incubated for 1 hr. at 37° in presence of 0·5 % (w/v) glucose, 200 μl. glutamic acid/ml., and penicillin as below.

Penicillin present (units/ml.)	**Increase in internal glutamic acid concentration (μl./100 mg.)**
0	**618**
2	**627**
20	**560**
50	**536**

The action of penicillin on glutamic acid assimilation by growing cultures

Action of penicillin on growth. The growth of an inoculum of *Staph. aureus* strain D in medium A was prevented by the presence of 0·08–0·1 Oxford units penicillin/ml. For assimilation measurements it is necessary to have reasonably large amounts of cells, and so the effect was tested of adding penicillin to growing cultures of the organism. Fig. 1 shows the effect of adding various concentrations of penicillin to growing cultures of *Staph. aureus* D, the additions being made after growth had taken place for 3½ hr. at 37° in medium B. Growth was followed turbidimetrically; it does not follow that an increase in turbidity after the addition of penicillin is accompanied by a corresponding increase in cell numbers. It can be seen from Fig. 1 that the turbidity of the cultures continued to increase normally for 30 min. after the penicillin additions. The turbidity continued to increase for 1–2 hr. according to the penicillin concentration added, smaller concentrations taking longer to bring the turbidity to a steady level than higher ones. Ten units penicillin/ml. bring about cessation of growth in about 1 hr., but 0·1 unit/ml. takes nearly 3 hr. to become completely inhibitory. Viable cell counts were carried out on many of the samples. It was found that approximately one division per cell takes place within the 30 min. following the addition of penicillin in any of the concentrations tested. There was then a steady loss of viability lasting over a period of hours and depending upon the penicillin concentration. The viable count had fallen to about 4 % of its value at 3½ hr. within 2 hr. of the addition of 10 units/ml., and to about 80 % within 2 hr. of the addition of 0·1 unit/ml. Lysis of the cells set in 3–4 hr. after penicillin addition, but general lysis did not occur with this organism for 24–30 hr. after penicillin addition. The findings described here for the effect of penicillin on growth are in general agreement with those of previous authors (Chain & Duthie, 1945).

Effect of penicillin on internal accumulation of glutamic acid. The accumulation of glutamic acid and lysine within the internal environment of *Staph.*

aureus cells growing in medium A before and after penicillin addition was next tested. The organism was inoculated into media at 37° and cells harvested at intervals throughout the growth period for assay of their internal amino-acids as previously described (Gale, 1947*a*). Penicillin (4 units/ml.) was added to half the culture at 4 hr. Fig. 2 shows the effect of the penicillin on growth and on the accumulation of glutamic acid within the cells. In the normal culture the amount of free glutamic acid within the cells increased during growth, reaching

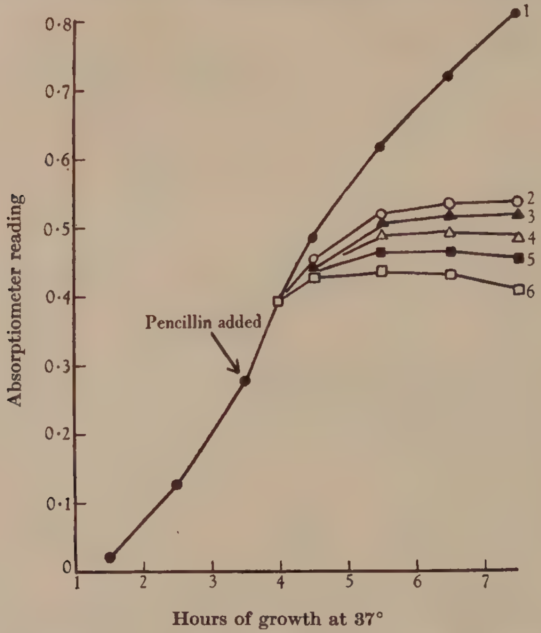

Fig. 1. Effect of addition of penicillin to growing cultures of *Staph. aureus*. Medium: salt mixture + 0·1% Marmite + 1·0% glucose. Penicillin concentrations added at $3\frac{1}{2}$ hr. Curve 1, no penicillin. Curve 2, 0·1 Oxford unit/ml. Curve 3, 0·5 Oxford unit/ml. Curve 4, 1·0 Oxford unit/ml. Curve 5, 5·0 Oxford units/ml. Curve 6, 10·0 Oxford units/ml.

a steady value as growth ceased. The total assimilation of glutamic acid/100 mg. cells was approximately constant throughout the growth period (Gale & Mitchell, 1947), but since the level measured inside the cell represents a balance between the amount withdrawn from the external environment and the amount metabolized, this level will be lowest when the internal metabolism is highest, i.e. when growth is taking place most rapidly (Gale, 1947*b*). After the addition of penicillin to the external medium, the accumulation of free glutamic acid within the cells increases normally for approximately an hour and then begins to decrease rapidly although growth has not ceased. It can be seen from Fig. 2 that the fall in the internal glutamic acid level continued until the culture ceased to grow. The cells were assayed for lysine content at the same time as their glutamic acid content, but, although the accumulation of lysine in the normal cells shows a rising curve of the same shape as that shown in Fig. 2 for glutamic acid, the addition of penicillin had no significant effect upon the accumulation of lysine within the cells. These results suggest that the cells ceased to assimilate glutamic acid, but not lysine, from the external medium shortly after the addition of penicillin to the medium.

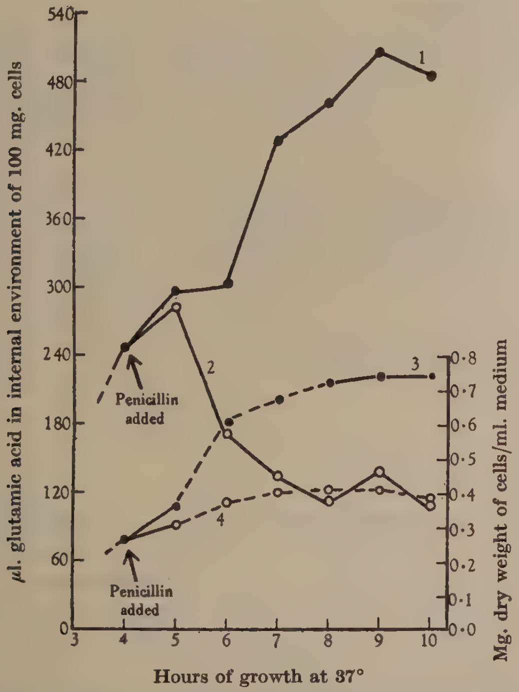

Fig. 2. Effect of addition of penicillin to growing cultures of *Staph. aureus* on the accumulation of free glutamic acid in the internal environment of the cells. Medium: casein digest + 0·1% Marmite + 1% glucose. Curve 1, accumulation of free glutamic acid in 100 mg. cells during normal growth. Curve 2, accumulation of free glutamic acid in 100 mg. cells during growth when penicillin (4 Oxford units/ml.) added at $3\frac{1}{2}$ hr. Curves 3 and 4, growth curves normal (3) and penicillin added at $3\frac{1}{2}$ hr. (4) to compare with curves 1 and 2 respectively.

Effect of penicillin on glutamic assimilation by growing cells. The assumption that glutamic acid assimilation is impaired after the addition of penicillin to the culture was first tested on growing cells. The experiment recorded in Table 3 shows that in the normal culture, the internal content of glutamic acid was 387 μl. higher in the cells (100 mg.) which had grown in the presence of added glutamic acid than in the cells grown in the 'deficient' medium B. In the cultures to which penicillin was added 1 hr. before glutamic acid, the difference between the two groups of cell was decreased to 116 μl. glutamic acid, and this difference was still further diminished to 49 μl. in the cultures to which penicillin was added 2 hr. before the glutamic addition. This again suggests that the capacity to assimilate and concentrate glutamic acid is impaired in cells growing in the presence of penicillin.

Table 3. *Effect of penicillin on glutamic acid assimilation by growing culture of* Staphylococcus aureus *D*

Staph. aureus D was inoculated into deficient medium B incubated at 37°. After 5 hr. (B) or 4 hr. (C) the culture was divided into two and penicillin (10 units/ml.) added to one half; after a further 1 hr. (B) or 2 hr. (C) each batch of culture was again divided into two and glutamic acid (200 μl./ml.) added to half of each batch; all cultures were then allowed to grow for a further hour and all were harvested 7 hr. after inoculation.

			Internal glutamic acid	
Culture	Penicillin 10 units/ml. added after time indicated	Glutamic acid (200 μl./ml.) added after time indicated	μl./100 mg. cells	Increase due to added glutamic. acid (μl.)
A	—	—	365	387
	—	6 hr.	752	
B	5 hr.	—	202	116
	5 hr.	6 hr.	318	
C	4 hr.	—	206	49
	4 hr.	6 hr.	255	

Glutamic acid assimilation by cells grown in the presence of penicillin

Since the assimilation of glutamic acid by cells growing in the presence of penicillin was apparently impaired, the ability of such cells to assimilate glutamic acid after removal from the growth medium was next tested. The cells were grown as usual in medium B and penicillin (10 units/ml. medium) added at 3½ hr. after inoculation. At 4½ hr. the cells were harvested and suspended in glutamic acid glucose salt solution as usual and their ability to concentrate glutamic acid in the internal environment compared with normal cells grown for the same period but in the absence of penicillin. Normal cells assimilated 638 μl. glutamic acid/100 mg., while the cells grown in the presence of penicillin assimilated 42 μl./100 mg. The addition of penicillin (50 units/ml.) to the external environment during the assimilation procedure had no significant effect. Washing the cells grown in presence of penicillin with distilled water or incubation with cysteine (1 mg./ml.) did not alter the impaired assimilation of glutamic acid. The assimilation of glutamine was of the same order as that of glutamic acid and was impaired to the same extent in the cells grown in penicillin.

Effect of penicillin concentration and time of contact. Reference to Fig. 1 shows that the speed with which penicillin brought about cessation of growth varied with the concentration of penicillin added to the culture. Table 4 shows the glutamic acid assimilated by cells harvested at intervals after the addition of various concentrations of penicillin to the cultures. The ability of these cells to assimilate glutamic acid was investigated as usual. Cells taken from cultures to which no penicillin had been added during growth assimilated 560–700 µl. glutamic acid/100 mg. under the standard conditions of test. Within 30 min. of the addition of penicillin (10 units/ml.) to the culture, the assimilatory power of the cells had fallen to 14 % of that of the control; within 1 hr. the assimilation had fallen to 4 % that of the control, and after 90 min. assimilation was no longer possible. Lower concentrations of penicillin produced the same failure of assimilatory power but took longer to make this complete; thus penicillin at 1 unit/ml. took over 2 hr. to prevent glutamic acid assimilation completely. If these values for assimilation are compared with the curves shown in Fig. 1, it can be seen that there is a correlation between the cessation of cell growth and the inhibition of glutamic acid assimilation.

Table 4. *Effect of the presence of penicillin during growth on the assimilation of glutamic acid by* Staphylococcus aureus

Cells grown in medium B and penicillin added in all cases after 3½ hr. growth at 37°. Cells harvested at various times after the penicillin addition, incubated for 1 hr. at 37° in glutamic acid (200 µl./ml.) and glucose (0·5 % w/v), and the increase in the internal glutamic acid content assayed as usual.

Penicillin concentration (units/ml. medium)	Glutamic acid assimilated (µl./100 mg. cells) Time of harvesting after penicillin addition				
	30 min.	1 hr.	1½ hr.	2 hr.	3 hr.
0	561	702	602	590	614
0·1	—	—	—	130	—
0·5	—	—	—	113	—
1·0	—	—	87	—	0
5·0	—	—	0	—	0
10·0	82	31	0	0	0

Effect of penicillin on general metabolic activity of cells

Table 5 shows the general metabolic activities of normal cells compared with those of cells harvested after 90 min. growth under the usual conditions in the presence of penicillin at 10 units/ml.

Table 5. *Metabolic activities of normal* Staphylococcus aureus *cells and of cells grown in presence of penicillin*

'Penicillin cells' grown for 90 min. in medium containing 10 units penicillin/ml.

	Normal cells	'Penicillin cells'
Respiration Q_{O_2}	21·5	19·6
Glucose oxidation Q_{O_2}	86·5	84·5
Glucose fermentation $Q_{CO_2}^{acid}$	96	108
Lysine assimilation (µl./100 mg.)	90	96
Glutamic acid assimilation	602	0
Comparative viable count	452	9

Respiration. This was measured in Warburg manometers containing 1·0 ml. suspension of cells and 2·0 ml. M/15 phosphate buffer pH 7·5 in the main cup, and 0·3 ml. 10 % (w/v) NaOH in the centre pot. The steady rate of respiration measured over 15 min. was determined in this manner and also in the presence of penicillin (50 units/ml.). The values given in Table 5 show that the respiration of the cells grown in the presence of penicillin was slightly lower than that of normal cells; in neither case did the addition of penicillin to the Warburg cup have any inhibitory effect on respiration.

Glucose oxidation. This was also measured in Warburg manometers in parallel with the blank respiration experiment; in this case 0·2 ml. 2 % (w/v) glucose was added from the side-bulb at the beginning of the experiment and the steady rate of oxygen uptake measured. There was no significant difference between the rates of oxidation carried out by the two cultures, and again the addition of penicillin to the manometer cups had no inhibitory effect.

Glucose fermentation. Manometers were assembled containing 1·0 ml. cell suspension and 1·5 ml. M/40-$NaHCO_3$ in the main cup, and 0·5 ml. 1 % (w/v) glucose in the side-bulb. The manometers were filled with a gas mixture containing 5 % CO_2 + 95 % N_2. After equilibration at 37° the glucose was added to the main reaction compartment and the rate of fermentation determined from the CO_2 evolution. Since the organism carries out a homolactic fermentation, the Q_{CO_2} measured in this way is a direct measure of lactic acid production. There was no significant difference between the rates of fermentation of the two cultures and the addition of penicillin had no inhibitory effect. The assimilation of glutamic acid in the experiments described is dependent upon the supply of energy from glycolysis; these measurements show that the impairment of assimilation is not a consequence of inhibition of fermentation by penicillin.

Lysine assimilation. This was measured, as previously described, by standing the deficient cells in a solution of 200 μl. lysine/ml. for 1 hr. at 37° and measuring the increase in the internal lysine concentration. The amount of lysine taken up is dependent upon the electrical properties of the cells (Gale, 1947 *a*) and on the intact nature of the cell wall (Gale & Taylor, 1947). Table 5 shows that the cells from the two cultures were essentially similar in their capacity to assimilate lysine and confirms the earlier finding that the accumulation of lysine within growing cells is independent of the presence of penicillin in the medium.

Comparative viable counts. Viable counts were carried out on standard volumes (of equal turbidity) of the two cell suspensions diluted 10^6 times by serial dilution. Table 5 shows that the viability of the suspension harvested from the medium containing penicillin was about 2 % of the normal cell suspension.

Penicillin is known to have four effects on *Staph. aureus* when added to growing cultures: (1) the cells become non-viable (Chain & Duthie, 1945); (2) their respiration progressively fails (Chain & Duthie, 1945; Hirsch, 1943–4); (3) lysis occurs after several hours (Fleming, 1929; Chain & Duthie, 1945), and (4) assimilation of glutamic acid is prevented. The results recorded in Table 5 show that the failure of glutamic acid assimilation preceded the failure of respiration and the onset of general lysis and would appear to take place simultaneously with or before loss of viability.

Effect of penicillin on internal metabolism of glutamic acid

In the previous paper (Gale & Mitchell, 1947) it was shown that some metabolism of glutamic acid took place after it had passed into the internal environment of the cell. This metabolism could be demonstrated since, if the amount of glutamic acid which accumulated inside the cell during assimilation was compared with the amount which was removed from the external environment, there was on balance a 'disappearance' of the amino-acid. This disappearance or metabolism of glutamic acid is inhibited by the triphenylmethane dyes. Table 6 shows the results of such balance experiments carried out during the assimilation of glutamic acid by washed suspensions of *Staph. aureus* cells both normal and harvested from medium to which penicillin (10 units/ml.) was added 90 min. prior to harvesting. In both cases glutamic acid disappeared during the assimilation experiment, the amount disappearing in the experiment with penicillin-treated cells being about 80 % of that disappearing with normal cells. However, with the normal cells the amount of glutamic acid in the internal environment increased by 370 μl. during assimilation while 831 μl. were withdrawn from the external medium. With the penicillin-treated cells the amount of glutamic acid withdrawn from the external medium was very much less, a decrease of only 106 μl. being measured, and the experimental error in the determination was of the order of 40 μl.; but the internal glutamic acid level decreased by 263 μl. The figures suggest that the internal metabolism of glutamic acid continued normally in the penicillin-treated cells, but since the passage of glutamic acid across the cell wall was blocked, this metabolism took place at the expense of the internal glutamic acid already present. In the normal cells the glutamic acid metabolized within the cells is balanced by assimilation from the external medium and an increase in the internal level to maintain equilibrium with the external concentration. The fall in the curve (Fig. 2) showing glutamic acid accumulation within the growing

Table 6. *Effect of penicillin on glutamic acid metabolism*

Organism grown in medium B. Penicillin 10 units/ml. added to half of culture 90 min. before harvesting. Cells centrifuged down, washed and suspensions incubated in glutamic acid solution + 1 % (w/v) glucose for 1 hr. at 37°. Internal and external glutamic acid assayed before and after incubation.

	External environment	Internal environment	Change in external environment	Change in internal environment	Glutamic acid metabolized
	(μl. glutamic acid)				(μl.)
Normal cells:					
Initial	1586	690	−831	+370	461
Final	755	1060			
Penicillin-treated cells:					
Initial	1646	579	−106	−263	363
Final	1540	815			

cells after the addition of penicillin to the medium is thus explained, since the assimilation of glutamic acid is blocked shortly after the addition, and metabolism of the internal glutamic acid then produces the drop in its internal concentration. These experiments provide further proof that the metabolism of glutamic acid by these cells is an intracellular process.

Effect of penicillin on glutamic acid assimilation by Streptococcus faecalis *ST*

Strep. faecalis ST needs about 10 times the concentration of penicillin required by *Staph. aureus* in order to prevent growth. When tested under the usual conditions, normal *Strep. faecalis* cells assimilated 142 μl. glutamic acid/100 mg. cells; cells which had been grown in the presence of penicillin (10 units/ml.) medium for 90 min. prior to harvesting assimilated 57 μl./100 mg. under the same conditions. The action of penicillin was thus to impair glutamic acid assimilation in these cells as in *Staph. aureus.*

DISCUSSION

The experiments described show that one action of penicillin on growing staphylococci is to prevent the assimilation of glutamic acid. Since it has been possible to study the assimilation of only such amino-acids as can be estimated by the decarboxylase technique, it is not possible at present to say whether this impairment of assimilation extends to amino-acids other than glutamic acid. The assimilation of lysine is certainly not affected in the same way, but this amino-acid is not assimilated by the same type of mechanism as glutamic acid. The Gram-positive cocci are, in general, nutritionally exacting for a range of amino-acids including glutamic acid, and it is reasonable to suppose that a loss of viability would follow inhibition of assimilation of these amino-acids. It is difficult, however, to distinguish between cause and effect, and it is not possible to say at present whether cells rendered non-viable by penicillin acting in some other way would be able to assimilate glutamic acid or not. There is a suggestion in the data shown in Table 4 and Figs. 1 and 2 that cessation of assimilation precedes loss of viability. In Fig. 2 there is definitely an increase in turbidity after the point at which the internal concentration of glutamic acid begins to fall instead of to rise, and the internal concentration does not reach a new steady level until growth (as measured by turbidity increase) ceases. The glutamic acid which accumulates inside the cell acts as a reservoir of amino-acid for protein synthesis (Gale, 1947*b*) and for other metabolic purposes (Gale & Mitchell, 1947), and it may be that growth will continue as long as there is more than a certain limiting concentration within the cell. In that case the sequence of events would be: (i) penicillin prevents the passage of glutamic acid into the cell; (ii) the synthesis of protein, etc., proceeds at the expense of the accumulated glutamic acid (and other amino-acids) within the cell and consequently the concentration falls; (iii) the internal concentration falls to the lowest level permitting synthesis of protein and growth ceases. Once penicillin has acted on the cell wall, the further growth is therefore limited by the amount of essential amino-acids accumulated within the cell, and it may also be the case that this suffices for sufficient growth to produce enlarged forms of the cells but not for complete division.

Penicillin has no effect on the mechanism whereby glutamic acid is assimilated and concentrated within the internal environment in normal 'resting' cells, but affects cells during growth in such a way that assimilation is prevented. This suggests that penicillin either combines with or produces a reorganization of the cell wall such that the assimilatory mechanism is blocked.

A recent paper by Shwartzman (1946) has shown that the resistance of Gram-negative organisms to penicillin is increased by the presence of certain amino-acids, aspartic and glutamic acids being very active in this respect. It is possible that a mechanism similar to that discussed above is also operative in this case.

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The Assimilation of Amino-acids by Bacteria

6. The Effect of Protein Synthesis on Glutamic Acid Accumulation and the Action thereon of Sulphathiazole

By E. F. GALE

Medical Research Council Unit for Chemical Microbiology,
Biochemical Laboratory, Cambridge

SUMMARY: The level of free glutamic acid accumulating within cells of certain Gram-positive cocci is lower in growing cells than in 'resting' cells, other conditions being equal. Part of the glutamic acid assimilated by growing *Staphylococcus aureus* is condensed into peptides or proteins, thus accounting for this apparent drop in glutamic acid accumulation. Sulphathiazole interferes with this condensation of glutamic acid into peptide form.

When Gram-positive cocci are grown in a medium deficient in free glutamic acid and then suspended in a solution of glutamic acid and glucose, glutamic acid is assimilated and concentrated in the internal environment of the cells. As the amino-acid is assimilated a portion of it undergoes metabolism, and the level of free glutamic acid attained inside the cell represents a balance between the rate at which it enters the cell and the rate at which it is metabolized inside the cell. When assimilation is studied in washed suspensions of cells, the metabolism of glutamic acid inside the cell does not lead to peptide formation but consists of some other form of alteration of the free glutamic acid molecule (Gale & Mitchell, 1947). The results presented in this communication show that in growing cells there is a further metabolism which results in peptide and protein formation and that this form of metabolism is inhibited by sulphathiazole.

Methods and organisms. The methods of estimation of glutamic acid, growth and assimilation have been described in the previous papers of this series (Gale, 1947). Two organisms have been used: *Staphylococcus aureus* 6773 isolated by Dr E. Topley and found by her to be sensitive to sulphathiazole at 1 mg./100 ml. concentration, and *Streptococcus faecalis* ST, used in the previous studies. Three growth media have been employed:

Medium A: tryptic digest of casein + 0·1 % (w/v) Marmite + 1 % (w/v) glucose.

Medium B: Stephenson's inorganic salts (Stephenson, 1939) + 0·1 % (w/v) Marmite + 1 % (w/v) glucose.

Medium C: 1 % (w/v) peptone (Difco) + 1 % (w/v) glucose.

Assimilation of glutamic acid by growing and resting cells of Streptococcus faecalis

The curves of Fig. 1 represent the internal concentration of glutamic acid/100 mg. dry weight of *Strep. faecalis* cells when these cells are tested at different ages under the various conditions indicated. Gale & Mitchell (1947) have shown that curve 3 represents the assimilation of glutamic acid in the absence of internal metabolism, and the curve suggests that the capacity to assimilate this amino-acid is roughly constant throughout the growth period but falls rapidly soon after growth ceases. Curve 2 represents the balance between the rate of assimilation (or entry into the cell) and the rate of metabolism of glutamic acid in washed non-growing cells, so that the difference between curves 2 and 3 for each culture represents the proportion of assimilated glutamic acid undergoing metabolism (other than peptide formation) inside the cell.

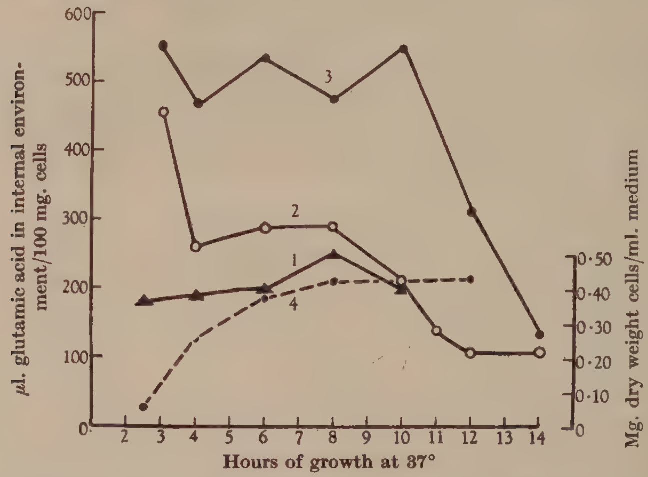

Fig. 1. Internal concentration of free glutamic acid in *S. faecalis* cells. Curve 1, cells grown in medium A (approx. 200 µl. free glutamic acid/ml.), harvested from the growing culture, washed and internal glutamic acid level assayed immediately. Curve 2, cells grown in medium B, harvested, washed, incubated for 1 hr. at 37° in glutamic acid (200 µl./ml.) and glucose (0·5%). Curve 3, cells grown and harvested as in 2 but incubation carried out in presence of crystal violet (1:10,000). Curve 4, growth curve in medium A.

Curve 1 shows a further decrease in the balance of free glutamic acid during the early stages of growth. Since the cells used to obtain curve 1 were growing but those for curve 2 were 'resting', it is possible that the difference between curves 1 and 2 represents, for each culture, the proportion of assimilated glutamic acid which is undergoing condensation into protein. It can be seen that curves 1 and 2 meet at that age of culture when active cell growth ceased, as judged turbidimetrically, and it has already been shown that peptide formation does not take place in non-growing cells of this nature (Gale & Mitchell, 1947). In the growing cells (curve 1) the level of free glutamic acid on balance rose as growth proceeded, suggesting that the proportion of assimilated glutamic acid entering into combination was greater in the early stages of growth. This aspect of assimilation can be studied more easily in *Staph. aureus*, since this organism accomplishes a higher concentration of glutamic acid in the internal environment than *Strep. faecalis* and consequently effects of this nature are of greater magnitude.

Accumulation of free glutamic acid inside growing Staphylococcus aureus *cells*

Fig. 2 shows the levels of glutamic acid attained inside cells of two strains of *Staph. aureus* at various times during growth in medium A. The curves are of the same nature as curve 1 in Fig. 1 and show the same general increase in concentration during growth. It can be seen that when growth was taking place most rapidly, between 5 and 6 hr., the accumulation of free glutamic acid was temporarily checked, again suggesting that this level within the cell is a balance between the rate of entry and the rate of combination into protein, etc. The same type of curve is given when growth and assimilation are followed in medium B and, for reasons explained below, this medium has been used for many of these studies. Fig. 1 shows that, in the case of *Strep. faecalis*, the amount of glutamic acid assimilated (i.e. withdrawn from the external environment) was approximately constant during the period of growth, and if the suggestion be true that the difference between curves 1 and 2 (Fig. 1) is due to condensation of free glutamic acid into protein or peptides, then it should be possible to show a variation in the ratio of combined/free glutamic acid at various stages of the growth period.

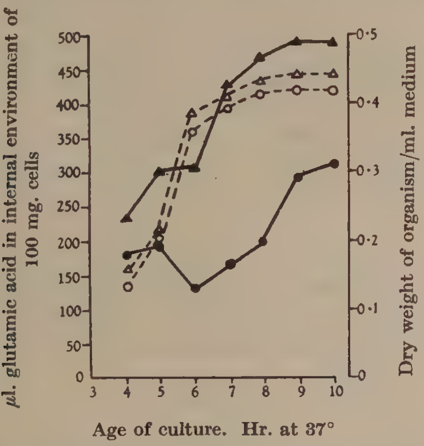

Fig. 2. Growth and accumulation of free glutamic acid in internal environment of *Staph. aureus*. Medium: casein digest + 2% glucose + 0·1% Marmite. Growth: strain A, ○---○, strain D △---△; glutamic acid: strain A ●—●, strain D, ▲—▲.

To test this *Staph. aureus* 6773 was grown in medium B in Roux bottles lying flat, all inoculated with a standard inoculum, and suitable amounts of the culture were harvested at various times during growth. The harvested cells were washed and the external, total and internal free glutamic acid assayed on samples as usual (Gale, 1947); the rest of the cells were then subjected to 20 hr. hydrolysis in boiling 5 N-HCl, the excess acid removed *in vacuo* and the total glutamic acid (free + that released from protein by hydrolysis) assayed. The ratio of the combined/free glutamic acid in the internal environment could then be calculated. Table 1 shows a series of results obtained in such a manner. It can be seen that: (1) the free glutamic acid content of the cells rose during growth, as shown before; (2) the combined glutamic acid/100 mg. dry weight of cells fell during growth; (3) the ratio of combined/free glutamic acid fell from 5·6 for 3 hr. culture to 1·5 for 8 hr. culture; (4) the total (free + combined) glutamic acid/100 mg. cells was approximately constant throughout the growth period. It follows from these results that the difference between curves 1 and 2 Fig. 1 (or their equivalent for *Staph. aureus*) was due to protein or peptide synthesis and that glutamic acid after assimilation into the growing cells was partly incorporated into protein, etc.

Table 1. *Free and combined glutamic acid content of growing* Staphylococcus aureus *cells*

		Glutamic acid			
Age of culture (hr.)	Growth (mg. dry wt. cells/ml.)	Free	Total (µl./100 mg. dry wt. cells)	Combined	Ratio combined/free
3	0·07	160	1050	890	5·58
4	0·127	188	981	793	4·23
4½	0·164	215	1083	868	4·04
5	0·179	289	1116	827	2·86
6	0·232	371	1147	776	2·09
7	0·264	405	1134	729	1·80
8½	0·302	405	1003	598	1·48

Effect of sulphathiazole on protein formation

Sensitivity to sulphathiazole. Staph. aureus 6773 was stated by Dr E. Topley to be sensitive to sulphathiazole at 1 mg./100 ml. When tested in medium C sulphathiazole at 10 mg./100 ml. was necessary to prevent the growth of an inoculum of 10^6 cells/ml. Since it was desirable to deal with reasonably large amounts of cells, the effect of adding sulphathiazole to the medium 1 hr. after inoculation was tested. In medium C 100 mg. sulphathiazole/100 ml. brought the growth to a stop after 5 hr., when the crop was approximately 60 % of that in the control culture; in medium B 100 mg. sulphathiazole/100 ml. slowed the growth so that the final crop was 80 % of the control, but the two cultures ceased active growth together; in medium A it was not possible to demonstrate any significant action of sulphathiazole under these conditions. The three media differ mainly in their free amino-acid content; the internal concentrations of free glutamic acid attained in 100 mg. of cells at the end of growth in the three media (sulphonamide-free) were: A, 500 µl.; B, 405 µl.; C, 195 µl.

Absence of effect of sulphathiazole on glutamic acid assimilation by washed cells. Cells harvested from medium B were incubated in the presence of glutamic acid (200 µl./ml.) and glucose (0·5 %) as usual, and the increase in the internal level of free glutamic acid assayed as previously described (Gale, 1947). Sulphathiazole even in saturated solution had no effect on the assimilation process under these conditions.

Effect of sulphathiazole on glutamic acid accumulation in growing cells. The organism was cultivated in medium B and sulphathiazole added to portions of the culture after inoculation; later the cells were harvested and their internal glutamic acid level assayed. Table 2 shows that the presence of sulphathiazole in the growth medium increased the amount of glutamic acid accumulating in the cells at the time of harvesting. Previous experience with the action of triphenylmethane dyes on assimilation (Gale & Mitchell, 1947) showed that an increase in the level of glutamic acid inside the cell might be attributable to an inhibition of some metabolic process involving glutamic acid inside the cell. Since sulphathiazole had no effect on assimilation in washed cells, it does not affect the metabolic processes blocked by crystal violet under similar circumstances. However, the results given in Table 2 are shown only by growing cells and the work described here has shown that there is a further form of metabolism in such cells which results in condensation of free glutamic acid into a combination, presumably of peptide nature. The question arises whether the increased levels of free glutamic acid in the presence of sulphathiazole are attributable to disorganization of this protein synthesis.

Table 2. *Effect of sulphathiazole on accumulation of glutamic acid in internal environment of growing* Staphylococcus aureus

All cultures inoculated with *Staph. aureus* 6773 at time 0 and incubated at 37°. Sulphathiazole added as below at 1 hr., all cells harvested at 4½ hr. and washed before assay.

Sulphathiazole content of growth medium (mg./100 ml.)	Growth at harvesting (mg. dry weight of cells/ml.)	Glutamic acid in internal environment (μl./100 mg. dry wt. cells)
0	0·146	198
1	0·135	280
10	0·121	306
100	0·102	325

Fig. 3 shows the accumulation of free glutamic acid within cells growing in medium C in the presence and absence of sulphathiazole. It can be seen that whereas the curve has the normal shape for the accumulation of glutamic acid in cells growing in the absence of sulphathiazole, the curve obtained for the cells growing in the presence of sulphathiazole is approximately a straight line coming at a level slightly below that attained by the normal cells at the end of growth.

Table 3 shows the ratio of free and combined glutamic acid determined during growth of the organism in the presence of sulphathiazole in medium B for purposes of comparison with Table 1. The ratio is approximately constant over the period studied. It has been shown in the early part of this paper that the variation of the level of free glutamic acid in growing cells is due to protein formation such that the lower the level, the greater the rate of protein synthesis. The data presented in Fig. 3 and Table 3 show that the normal condensation of glutamic acid into protein becomes disorganized in the presence of sulphathiazole. It does not, of course, follow that sulphathiazole interferes directly with the condensation of glutamic acid into peptide, as any interference or inhibition of protein synthesis would be expected to produce the results described

Table 3. *Free and combined glutamic acid content of* Staphylococcus aureus *growing in medium B+100 mg. % sulphathiazole*

Age of culture (hr.)	Growth (mg./ml.)	Glutamic acid (μl./100 mg. dry wt. cells)		Ratio combined/ free	Ratio for cells of same age in control culture without sulphathiazole
		Free	Combined		
3	—	—	—	—	5·58
4	0·09	284	821	2·89	4·23
4½	0·110	379	834	2·20	4·04
5	0·119	332	842	2·54	2·86
6	0·170	297	822	2·76	2·09
7	—	—	—	—	1·80

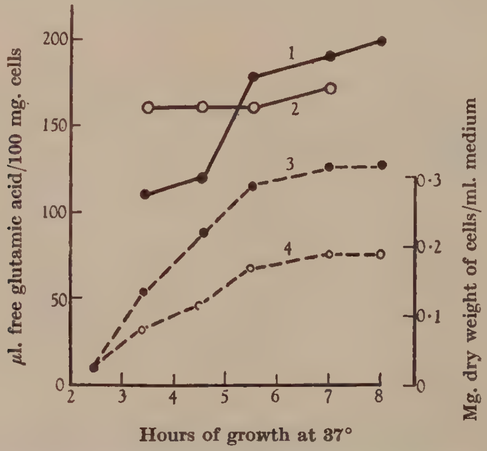

Fig. 3. Effect of sulphathiazole on accumulation of free glutamic acid in growing cells (*Staph. aureus*). Medium, 1% peptose + 1% glucose. Curve 1, glutamic acid accumulation in normal medium. Curve 2, glutamic acid accumulation in medium containing 100 mg. sulphathiazole % added at 1 hr. Curve 3, normal growth curve. Curve 4, growth curve in medium containing 100 mg. sulphathiazole %.

DISCUSSION

The work described in this series of papers can be summarized as follows:

(1) The passage of glutamic acid across the cell wall of certain Gram-positive bacteria requires energy and this can be supplied by exergonic metabolism such as glycolysis (Gale, 1947).

(2) The Gram-positive bacteria examined assimilate glutamic acid and concentrate it in the free state in the internal environment so that, at equilibrium, the internal concentration is greater than that in the external environment (Gale, 1947; Taylor, 1947).

(3) This ability to concentrate glutamic acid in the free state within the cell is apparently confined to Gram-positive species (Taylor, 1947).

(4) Within the cells, glutamic acid undergoes metabolic change. In growing cells, part of the assimilated glutamic acid is condensed into peptides or proteins. In non-growing as well as in growing cells, other forms of metabolism take place during assimilation (Gale & Mitchell, 1947).

(5) Tyrocidin and detergent substances release the glutamic acid from inside the cells by modification of the permeability of the cell wall (Gale & Taylor, 1947*a*), Tyrocidin causes an actual rupture or partial solution of the cell wall of *Strep. faecalis* (Mitchell & Crowe, 1947).

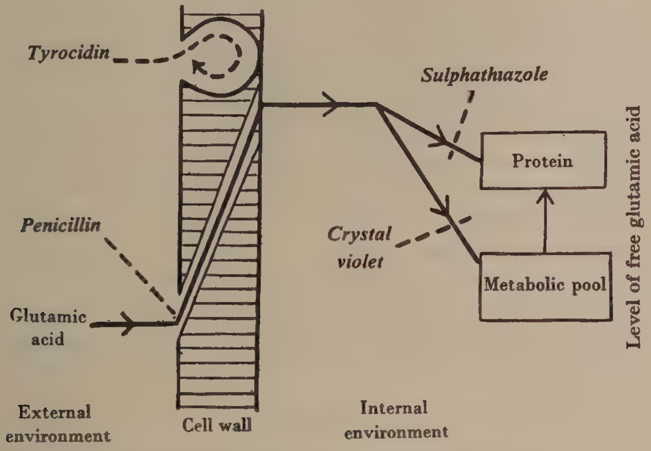

Fig. 4. Assimilation of glutamic acid by Gram-positive bacterial cell and action thereon of chemotherapeutic agents.

(6) The level of free glutamic acid attained within the cell depends upon (*a*) the external concentration and (*b*) the balance between the rate of entry into the cell and rate of metabolism within the cell. Consequently, any substance inhibiting the internal metabolism will give rise to an increased concentration of free glutamic acid within the cell while any substance interfering with the passage of the amino-acid across the cell wall will give rise to a decreased concentration within the cell.

(7) Penicillin prevents the passage of glutamic acid across the cell wall but does not interfere with the internal metabolism of glutamic acid. Penicillin acts in this way only if the cells concerned are grown for a short period in its presence (Gale & Taylor, 1947*b*).

(8) Triphenylmethane dyes inhibit the internal metabolism of glutamic acid in *Strep. faecalis* (Gale & Mitchell, 1947).

(9) Sulphathiazole interferes with the formation of protein, etc., from glutamic acid in growing cells.

These findings are represented diagrammatically in Fig. 4. The question arises as to how far the effects described for the action of various substances can explain the antibacterial action of these substances. All the substances tested so far are markedly more effective against the Gram-positive bacteria than the Gram-negative species but some of them, e.g. the dyes and sulphathiazole, are effective in high concentration against the latter. Taylor (1947) has shown that the concentration of free glutamic acid within the cells was a property only of the Gram-positive organisms examined, but it may be that the later stages of protein synthesis, etc., within the cell are common to all bacteria. In general Gram-negative bacteria are able to synthesize glutamic acid and consequently do not need to assimilate the free amino-acid, so that they would not be expected to be sensitive to the action of a substance whose only function was to prevent such assimilation. This may be the case with penicillin (see Gale & Taylor, 1947*b*). If the later stages of the assimilation process—peptide condensation and amino-acid interchange—are essentially similar in all bacterial cells then one would expect their sensitivity to inhibitors of these processes to depend upon the importance of the rates of the relevant reactions and the concentrations of relevant reactants in the cells. To understand the importance of assimilation processes with regard to dye and sulphonamide action, it will be necessary to determine how the anabolic processes in Gram-negative organisms are related to these disclosed for Gram-positive species. The relation between these processes and resistance to penicillin, the sulphonamides and dyestuffs also needs investigation. The action of tyrocidin and the detergents lies in a rupture of the cell membrane, and the release of glutamic acid etc. from the internal environment is therefore merely symptomatic of the release of all soluble cell-constituents (Hotchkiss, 1944).

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A Method for the Large-scale Production of Streptomycin by Surface Culture

By G. C. AINSWORTH, ANNIE M. BROWN, P. S. S. F. MARSDEN,
P. A. SMITH AND J. F. SPILSBURY

The Wellcome Physiological Research Laboratories, Beckenham, Kent

SUMMARY: Working details are given of a method for producing streptomycin by the surface culture of *Streptomyces griseus* in pint milk bottles on a papain digest of beef + meat extract + glucose + mineral salt medium. Streptomycin titres in the crude culture filtrates of 250 μg./ml. or more were obtained after 10–14 days' growth.

Evans's peptone, papain digest of spent pancreas from insulin manufacture, papain digest of yeast, and a proprietary casein-meat hydrolysate were found to be possible alternative sources of organic nitrogen. Growth was good on media containing peptic digest of beef or peptic-tryptic casein digest, but the streptomycin titre was low.

The utilization of glucose and the production of streptomycin depended on the relative amount of nitrogen present in the medium.

During the latter half of 1945 attempts were made in these laboratories by Mrs Ursula Wilson to produce streptomycin from *Streptomyces griseus* on the lines advocated by American workers (see review by Waksman & Schatz, 1945). Great difficulty was experienced in obtaining growth on the surface of the medium, the investigations were hampered by the lack of a streptomycin standard by which to measure the yield, and in spite of much experimentation the results were unsatisfactory. At the beginning of 1946 the work was continued by two of us (G. C. A. and A. M. B.) with indifferent results, until a particularly suitable medium for the growth of the organism was found and the conditions required for promoting good surface growth were recognized. At the same time, the streptomycin assay was standardized by the receipt of a sample of streptomycin sulphate from America. During the early summer the opportunity arose for a short period of experimental large-scale streptomycin production using the plant at the Wellcome Penicillin Unit. This paper summarizes aspects of the experimental work and outlines the production method devised.

METHODS AND MEDIA

Strain. The strain of *Streptomyces griseus* employed was Waksman's 'strain 4' (National Collection of Type Cultures no. 6961) but, because of an impression that the strain had undergone slight variation while in these laboratories, a culture of the organism actually used in the large-scale trials has been returned to the National Collection of Type Cultures where it is catalogued as no. 7187.

Method of assay. The method of assay for streptomycin, a dilution test against *Escherichia coli*, is described by Brown & Young (1947); it is only necessary to state here that the unit employed is 1·0 μg. of streptomycin base.

Belmont medium

The unsuccessful experiments on media and methods of culture will not be detailed. The best and most used of the earlier media was the one advocated by Waksman & Schatz (1945), which had the following composition: glucose 10·0 g.; NaCl 5·0 g.; peptone (Evans's)* 5·0 g.; meat extract (Wilson's or Lab Lemco) 5·0 g.; water to 1 l.; pH 7·0. Surface growth on this medium was frequently fair although the streptomycin titre was invariably low. At other times, for reasons still not fully understood, the growth was wholly submerged and streptomycin production negligible. Waksman emphasized the importance of meat extract and peptone for streptomycin production, and when these ingredients were substituted for casein hydrolysate in the Wellcome modification of Czapek-Dox solution, a synthetic medium devised for penicillin production by Dr C. G. Pope (Clayton, Hems, Robinson, Andrews & Hunwicke, 1944), it was at once apparent that a very favourable medium for the growth of *S. griseus* and the production of streptomycin had been found. The composition of this medium, which will be referred to as 'Belmont medium', was, in % (w/v): $NaNO_3$, 0·4; KH_2PO_4, 0·1; KCl, 0·05; $MgSO_4.7H_2O$, 0·5; $FeSO_4.7H_2O$, 0·005; $CuSO_4.5H_2O$, 0·00175; sodium citrate, 0·2; sodium acetate, 0·2; glucose, 4·4; peptone (Evans's), 1·0; meat extract (Wilson's), 0·5; water to 100 ml. The pH was adjusted to 7·0 with NaOH, and sterilization was effected by autoclaving for 15 min. at 15 lb./sq.in.

Glaxo flasks (Clayton *et al.* 1944) containing 200 ml. amounts of medium were found to be convenient culture vessels; later pint milk bottles were substituted for the Glaxo flasks except for cultures to be used as inoculum, when the larger surface area given in a Glaxo flask was advantageous.

Inoculation

The conidia of *S. griseus* are difficult to wet and tend to aggregate in clumps (Carvajal, 1946); this is probably the reason that, to establish good surface growth, a large inoculum and the addition of a wetting agent are essential. The routine procedure finally adopted was to add a 1 : 10^4 dilution of sterile 'calsolene'† to a freely sporing 7–14-day liquid culture, and after vigorous shaking to break up the mycelial mat, using the resulting suspension of spores and mycelial fragments as inoculum at the rate of 3–4 ml./200 ml. of the medium to be inoculated. By this method a film of surface growth was apparent after 24 hr. at 28°, and was complete after 48 hr.

Incubation temperature

Experiments showed 28° to be near the optimum for growth of *S. griseus* and for streptomycin production. At this temperature the surface of the medium was completely covered by the second day, and the thick wrinkled mycelial mat remained white. At 25° growth and streptomycin production were somewhat slower, but a satisfactory surface coverage and a final titre equal to that obtained at 28° resulted. At 31° the optimum had been passed; the surface of the medium became covered slightly more rapidly, but the thinner mycelial mat, at first greyish in colour, turned brown and became waterlogged and the streptomycin titre fell. The browning of the mycelium at higher temperatures could, however, be counteracted by lowering the temperature to the optimum or, better, to slightly suboptimal (25°).

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* Evans, Sons, Lescher and Webb Ltd., London and Liverpool.

† Obtained from Imperial Chemical Industries Ltd.

Using Belmont medium and the methods outlined above titres of 200–300 μg. streptomycin/ml. at 10–12 days were regularly obtained, and on occasion titres of over 400 μg./ml. resulted (see Table 1).

Table 1. *Streptomycin production on Belmont medium and analysis of metabolism fluid*

Period of incubation (days)	0	3	4	5	6	7
Streptomycin titre (μg./ml.)	0	18	45	95	105	215
pH	—	6·75	6·82	6·78	6·52	6·17
Composition of medium as % of original:						
Glucose	100	90·5	85·5	71·0	58·0	38·0
Total N	100	81·5	81·0	70·5	74·0	75·5
Amino-N	100	100·0	100·0	89·0	86·0	66·5
Ammonia-N	100	55·0	31·0	25·0	24·5	130·0

Period of incubation (days)	8	10	11	12	13	14
Streptomycin titre (μg./ml.)	305	390	445	360	345	360
pH	6·8	7·48	7·75	7·78	8·25	8·3
Composition of medium as % of original:						
Glucose	33·5	16·5	10·0	7·0	5·0	4·5
Total N	69·0	66·5	72·0	72·5	71·5	73·0
Amino-N	61·0	44·5	39·0	39·0	37·5	37·5
Ammonia-N	40·0	180·0	300·0	400·0	500·0	490·0

Experimental media

The prospect of a large-scale production trial in the penicillin plant necessitated modifications in the Belmont medium because of the difficulty and expense of obtaining sufficient peptone and meat extract under the prevailing conditions. Various *ad hoc* experiments were therefore undertaken to find substitutes for these ingredients, and at the same time a more systematic study of *S. griseus* was made to elucidate its nutritional requirements and to explain the success of Belmont medium. The results of the former experiments are indicated below and an account of the latter investigation is given by Spilsbury (1947).

Organic nitrogen. The effects on streptomycin production of various substitutes for Evans's peptone are summarized in Table 2. In general, the vigour of growth on the different media was directly correlated with the streptomycin titre, but although growth was quite heavy on media containing peptic digest of beef or peptic-tryptic casein digest, the titres were very low on such media. Papain digest of beef, digest of spent pancreas from insulin manufacture, papain digest of yeast, and 'Casydrol' (a casein-meat hydrolysate; Bengers Ltd., Holmes Chapel, Cheshire) all showed promise. Papain digest of beef was adopted for the production medium. In addition to the materials tabulated, a papain digest of *Penicillium* felts was tried but found worthless, and no success was obtained with cornsteep liquor either as a nitrogen source or in place of meat extract.

Table 2. *Effect on streptomycin production of replacing casein hydrolysate in Wellcome modification of Czapek-Dox solution by other organic nitrogen sources, with or without meat or yeast extract*

		Streptomycin titre (μg./ml.) at 10–12 days		
Nitrogen source	Equivalent total N (μg./ml.)		+0·5 % meat extract	+yeast extract
Casein hydrolysate 0·25 % (w/v)	0·4	90*	100	—
Peptone (Evans's) 1·0 % (w/v)	1·5	—	250–400†	—
Papain digest of beef (concentrated):				
10 % (v/v)	1·5	75	270	—
5 % (v/v)	0·75	65–70	150–250	—
2·5 % (v/v)	0·4	25–60	75–160	—
Peptic digest of beef:				
20 % (v/v)	0·86	40	—	—
10 % (v/v)	0·43	80	—	—
Spent pancreas digest	1·2	160	—	—
	1·06	105	—	160–200
	0·53	50	—	35–60
	0·21	30	—	20–55
Papain digest of yeast	1·0	190	240	—
	0·5	160	140	—
Papain digest of soya flour	1·2	150	150	—
	0·8	120	140	—
	0·4	40	100	—
Casein digests:				
Papain	—	25	—	—
Tryptic	—	25	—	—
Peptic-tryptic	1·06	30–50	110	—
'Casydrol':				
2·0 % (w/v)	2·6	100	—	—
1·0 % (w/v)	1·3	120–200	—	80–220
0·5 % (w/v)	0·65	45–100	—	45–55
0·25 % (w/v)	0·325	20–25	—	20–25

* Wellcome modification of Czapek-Dox solution. † Belmont medium.

Meat extract. The beneficial effect of meat extract on streptomycin production was repeatedly confirmed. For example, in a large factorial experiment, certain results of which are given in Table 4, the streptomycin titres at 12 days for 5 % papain digest + 4 % glucose with the addition of 0·5, 0·25 and 0·0 % meat extract were 150, 100 and 70 μg./ml. respectively. In a limited series of trials Marmite and lentil extract proved to be of no value as a replacement for meat extract, but some success was obtained with yeast extract (see Table 2).

LePage & Campbell (1946) used 1·0 % (w/v) Bacto yeast extract as a substitute for peptone and meat extract. The yeast extract used in the present work was prepared by adding 100 g. of dried yeast to 1 l. of boiling water, allowing to cool, and using the filtered liquid (which had a total N of *c.* 2·5 mg./ml.) at the rate of 1·0 or 2·0 % (v/v), i.e. at a total N level of 0·025 or 0·05 mg./ml. in the finished medium.

Table 3. *Streptomycin titre and glucose utilization at* 12 *days in Belmont and in Waksman's media*

	Initial concentration of glucose (%) in medium					
	4·0		2·0		1·0	
	Streptomycin (µg./ml.)	Glucose utilization (%)	Streptomycin (µg./ml.)	Glucose utilization (%)	Streptomycin (µg./ml.)	Glucose utilization (%)
Belmont medium	310	98	190	97·5	70	97·5
Waksman's medium	115	60	115	99	60	100

Table 4. *Streptomycin titre and glucose utilization at* 11 *days in Belmont medium with peptone replaced by papain digest of beef*

	Initial concentration of glucose (%) in medium					
	9·0		6·0		4·0	
Papain digest (%, v/v)	Streptomycin (µg./ml.)	Glucose utilization (%)	Streptomycin (µg./ml.)	Glucose utilization (%)	Streptomycin (µg./ml.)	Glucose utilization (%)
5·0	190	57	160	72·5	150	93·5
2·0	60	37	>70	51	75	80
1·0	40	33	40	42	<30	54

Carbohydrate. Most of the experimental work on the carbohydrate component of the medium was directed towards exploring the possibility of replacing glucose by lactose and ascertaining the most efficient glucose level. When lactose was substituted for all or part of the glucose in Belmont medium, or was combined with papain or cornsteep liquor, growth was poor and titres low. Glucose had a marked effect on streptomycin production. The results of experiments on varying the percentage glucose in Belmont and modified Belmont medium and in Waksman's medium are summarized in Tables 3 and 4.

Increasing the glucose in Waksman's medium from 1·0 to 2·0 % increased the streptomycin titre, but a further increase had no effect. Decreasing the glucose in Belmont medium had the reverse effect (see Table 3), and though 6·0 or 9·0 % glucose in modified Belmont medium increased the streptomycin titre the percentage glucose utilized decreased (see Table 4). It is clear from these results (and those of Spilsbury, 1947) that the C/N ratio is important. Nitrogen is the limiting factor in Waksman's medium containing 4·0 % glucose, and 9 % glucose is supra-optimal for the modified Belmont medium containing 2·0 or 1·0 % papain digest.

THE PRODUCTION PROCESS*

Inoculum

A culture of *S. griseus* (N.C.T.C. 7187) was subcultured on 2 % agar slopes of Belmont medium in 6 × 1 in. culture tubes. After 7 days' incubation at 28–29° the tubes were sealed by dipping the cotton plugs into molten paraffin wax and stored in the dark at 5° until required.

The medium used for the production of conidia (spore medium) was a modification of Moyer's medium, having the following composition: papain digest of beef (see below) 6·0 % (v/v); all % (w/v): molasses, 1·5; glycerol, 0·58; NaCl, 0·5; $FeSO_4.7H_2O$ (A.R.), 0·0015; $CuSO_4.5H_2O$ (A.R.), 0·009; $(NH_4) Mo_7O_{24}.4H_2O$ (A.R.), 0·0013; $MnSO_4.4H_2O$ (A.R.), 0·00028; KH_2PO_4, 0·006; $MgSO_4.7H_2O$, 0·005; water to 100 ml.

This medium was prepared in 60 l. batches in a steam-heated enamelled cauldron. After adjusting to pH 7·0 ± 0·2 by the addition of pellet sodium hydroxide (B.P. quality), the unfiltered medium was distributed in 300 ml. quantities into carefully washed 2·5 l. bottles of the Roux type (Thompson bottles), autoclaved for 15 min. at 15 lb./sq.in., and allowed to stand overnight before inoculation.

About 10 ml. of a 1 : 10,000 sterile aqueous 'calsolene' solution were added to a slope culture (6 × 1 in. tube) and the growth rubbed off the agar with the pipette. The whole of the suspension so obtained was used to inoculate one bottle of spore medium which was then incubated at 28·5°. After 7 days 3·0 ml. of 1 % 'calsolene' solution were added, the cotton plug replaced by a sterile rubber bung, and the bottle thoroughly shaken. The heavy spore suspension so obtained was used to inoculate a further fifty Thompson bottles of spore medium at the rate of 4·0 ml./bottle; the transfer being effected by means of sterile glass tubes (internal diam. approx. 5 mm.) plugged at one end with cotton-wool and calibrated to hold 4·0 ml. This larger batch of inoculated spore medium was also incubated for 7 days at 28·5° and then used as inoculum for the production medium. A small proportion of each batch was reserved as inoculum for more spore medium, and the process was repeated until it was considered necessary to revert to a sealed culture.

Production medium

The production medium was a modification of Belmont medium in which the peptone was replaced by papain digest of beef, the meat extract was decreased in quantity, and 10 p.p.m. of manganese substituted for the trace of iron (Spilsbury, 1947). It had the following composition (all % (w/v) unless otherwise stated): $NaNO_3$ (Chilean), 0·4; KH_2PO_4, 0·1; KCl, 0·05; sodium citrate, 0·2; sodium acetate (recryst.), 0·2; $MnSO_4.4H_2O$, 0·004; $CuSO_4.5H_2O$,

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* The large-scale production was carried out in the penicillin surface culture plant designed by Dr C. G. Pope and Dr W. B. Hawes; it is hoped that a fuller description of this plant will be published.

0·00175; glucose monohydrate, 4·0; Wilson's* pure extract of beef, 0·25 %; papain digest of beef (see below), 5·0 % (v/v); water to 100 %.

A papain digest of beef was prepared by the method used by Mr J. G. C. Campbell in the W.P.R.L. Media Production Unit:

> 120 lb. beef muscle, finely minced, trimmed from fat and suspended in 90 l. of water previously heated to 80° and contained in a steam-heated cauldron fitted with a 1 h.p. motor-driven propeller stirrer. The temperature was raised to 60°, 190 g. of powdered papain added, and the temperature kept constant for 4 hr. At the end of this period the digest was brought to the boil and boiled for 5 min., allowed to cool, and then filtered through a bag filter. The digest was usually prepared the day before it was required, and after storage at 5·0° overnight, any fat on the surface was removed by skimming. This operation was important because the thin film of fat which otherwise covered the surface of the final medium had an adverse effect on the growth of the actinomycete.

The whole of the digest from 120 lb. beef was incorporated in 1700 l. of medium, the ingredients for which were measured out and placed in a stainless steel mixing tank fitted with a motor-driven propeller stirrer. Water at a temperature of 80° was added to make up the required volume, 3 kg. of celite (Johns Manville Ltd.) was stirred in to aid the subsequent filtration, and the pH was adjusted to 7·0 with sodium hydroxide pellets. The hot medium was passed through two bag filters each containing a further 3 kg. of celite, cooled to 50°, and then stored in a stainless steel holding tank which supplied the filling machine.

Before autoclaving, the production medium gave a chemical analysis of the following type: total N, 2·3–1·8 mg./ml.; amino-N, 0·84–0·5 mg./ml.; ammonia-N, *c.* 0·05 mg./ml.; nitrate (as $NaNO_3$), 0·2 %; total reducing sugars (as glucose), 4·0–3·6 %; total solids, 80–60 mg./ml. The specific gravity was about 1·03.

After autoclaving a slight increase in reducing substances was noted, the pH fell to 6·3–6·2, and a deposit was observed along the bottom of the bottles.

Bottling and sterilization

Pint milk bottles ('crown cork milk bottles') were charged with production medium at the rate of 200 ml./bottle by means of an automatic bottle-washing and filling machine. After filling, the bottles were plugged by hand with non-absorbent cotton-wool, fitted with loose-fitting aluminium caps (Wellcome Penicillin Unit Type 2, see Pl. 1, fig. 2), and arranged in twelves in wire baskets to facilitate handling. The aluminium caps were those previously used for penicillin production, and they had been designed to permit a similar passage of air to that allowed by the cotton-wool plugs. When a closer fitting cap was tried it was found that the diminished aeration gave substantially lower titres.

The medium was very suitable for bacterial growth, and it was found necessary to plug as well as cap the bottles previous to inoculation because most contamination was found to occur during the cooling after autoclaving. Sterilization of the medium was effected by autoclaving at 2–5 lb./sq.in. for 30 min. This was not entirely satisfactory because on incubation a small amount of contamination by a *Bacillus subtilis*-like organism occurred in uninoculated, plugged and capped bottles. Autoclaving at 15 lb./sq.in. for 15 min. proved satisfactory, but extension of the time to 1 hr. rendered the medium useless for the growth of *S. griseus*.

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* Wilson's Meats Ltd., London.

After autoclaving, the baskets of hot bottles were packed on to the monorail carriers, which supported the baskets in an almost horizontal position, and passed through a cooling tunnel in which the temperature of the bottles was brought down to that of the incubators (28·5°) by a blast of air. The carriers were then removed to the spray-gun room.

Empty canisters with the spray-guns attached were returned to the spore preparation section where they were cleaned by rinsing in warm water and the cotton-wool air filters were renewed. Experience during the developmental stages showed that traces of detergents such as 'teepol' or soap and the use of dilute phenol solutions for rinsing out spray-guns after washing had a deleterious effect on the inoculum.

Inoculation

The spray-guns used to inoculate the production medium were Aerograph guns (type MP). Each gun was fitted with a cotton-wool air filter and a 1 l. canister containing a filter of 50/in. mesh wire gauze and connected to the gun by 6 ft. of rubber tubing. The nozzle of the gun was covered with a cap of cotton-wool which was tied in place and the whole gun put into a small calico bag. The canister lid was covered with a square of cotton cloth which was tied down round the canister. The gun and canister were then carefully packed into a specially constructed galvanized iron box and the whole sterilized by autoclaving for 1 hr. at 15 lb./sq.in.

The sterilized guns were taken to the sterile room where the canisters were filled with inoculum and from thence to the spray-gun room where the nipple of the air filter was attached to the air line (compressed air at 5 lb./sq.in.), the canister being suspended 2–3 ft. above the bottles to be inoculated. The gun was then removed from the bag and the nozzle flamed. The operator removed the metal cap from the bottle to be inoculated and discarded the cotton plug (see next section). The mouth of the bottle and the nozzle of the gun were flamed. Inoculum was injected into the bottle as a fine spray for 8 sec. The mouth of the bottle was again flamed and the cap replaced. The volume of inoculum introduced into each bottle was 3·0 ± 0·5 ml., and this amount was checked at intervals by similarly delivering inoculum into a 10 ml. measuring cylinder. It was found that 192 bottles could be inoculated from one canister containing about 900 ml. of inoculum by one person in 20 min.

Incubation

The inoculated medium was incubated for 10–14 days in two large incubators each 165 ft. long × 14 ft. wide and capable of holding four rows of thirty-three carriers (Pl. 1, fig. 1). The temperature was maintained at 28·5 ± 1·0° by air-conditioning plant.

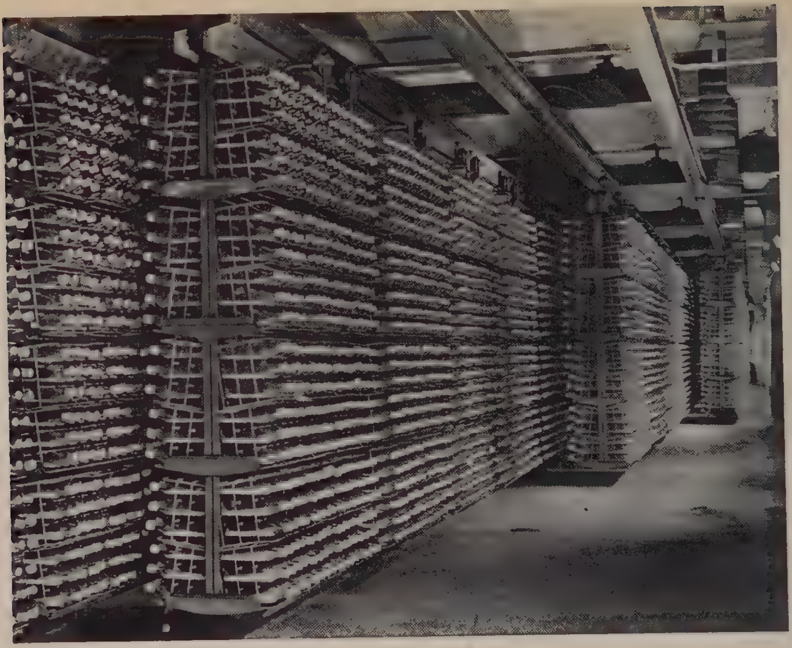

Fig. 1

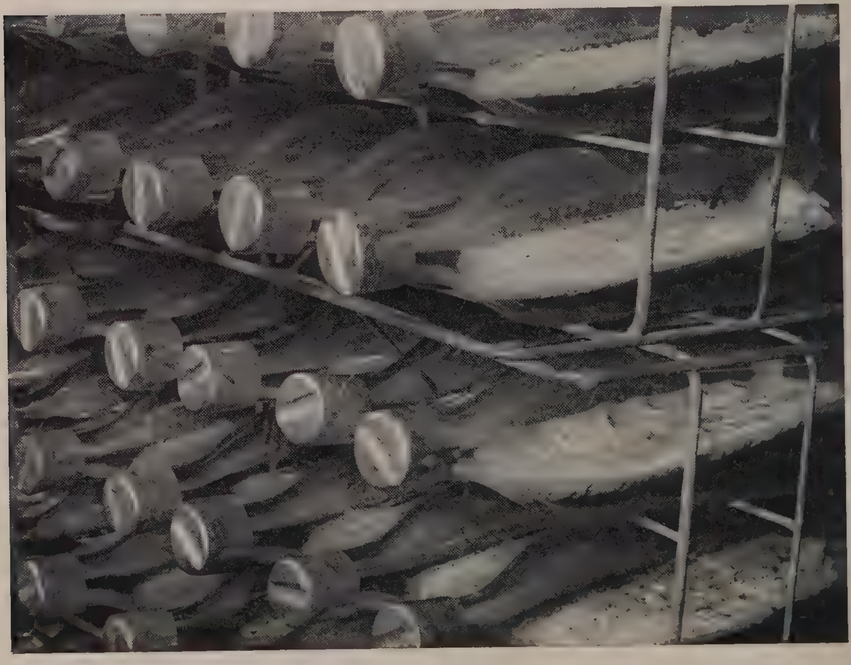

Fig. 2

G. C. Ainsworth, A. M. Brown, P. S. S. F. Marsden, P. A. Smith and J. F. Spilsbury—A method for the large-scale production of streptomycin by surface-culture. Plate 1

Harvesting

This was effected in a mechanical harvester in which bottles were inserted and drained of their contents for 3–4 min. before being transferred to the input end of the bottle-washing and filling machine. The crude filtrate was filtered and then pumped to the extraction department.

Extraction

The streptomycin was extracted from the culture fluid by a method involving charcoal adsorption, elution with dilute aqueous phosphoric acid, readsorption on charcoal followed by elution with acidified methanol from which the streptomycin was isolated as the hydrochloride. This process is described in detail in the next paper (Woodthorpe & Ireland 1947).

We wish to express our gratitude to our Beckenham colleagues, too numerous to mention individually, whose advice and help were always put freely at our disposal.

We are also indebted to all those laboratory technicians, and particularly to Mr J. Elson, Miss E. Howard, Mr Y. Jaulmes and Miss Jean M. Weston, who helped with the large amount of routine and assay work which this investigation involved.

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EXPLANATION OF PLATE

Fig. 1. General view of the incubator.
Fig. 2. Detailed view, showing bottles arranged in wire baskets, and methods of capping.

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A Method for Extracting and Purifying Streptomycin Suitable for Large-scale Production

By T. J. WOODTHORPE and D. M. IRELAND

The Wellcome Physiological Research Laboratories, Beckenham, Kent

SUMMARY: A method is described for extracting streptomycin from culture filtrates by adsorption on charcoal at pH 6–8, elution with 1·2% (v/v) aqueous phosphoric acid, readsorption of the eluate on charcoal at pH 7, elution with acidified methanol, followed by evaporation at reduced pressure and precipitation of streptomycin by dilution of the concentrated methanol eluate with 5 volumes acetone or amyl acetate. An indication is given of the order of recovery, and the potency of the product obtained. The stability of streptomycin under the conditions of pH and temperature to which it may be subjected during the extraction is outlined. The process has been carried out in pilot-scale production equipment handling 1500 l. batches of culture filtrate.

The aim of this work was to devise a method of extracting streptomycin from culture filtrates prepared by the method of Ainsworth, Brown, Marsden, Smith & Spilsbury (1947) which could be applied to the large-scale and economic manufacture of the antibiotic. In particular, a method was required that could be carried out in an extraction plant previously used for penicillin production.

Extraction methods given by other workers can be summarized as follows:

(1) A. Adsorption on charcoal at pH 7·0 and elution with 0·5% HCl in methanol (Waksman & Schatz, 1945; LePage & Campbell, 1946; Carter, Clark, Dickman, Loo, Skell & Strong, 1945).
   B. Concentration of neutral eluate (LePage & Campbell, 1946).
   C. Precipitation of streptomycin hydrochloride, by addition of a large excess of ether or acetone to the eluates or concentrates (Waksman & Schatz, 1945; LePage & Campbell, 1946; Carter *et al.*, 1945).

(2) Charcoal adsorption at low pH to remove pigments followed by extraction of the streptomycin as in 1 (LePage & Campbell, 1946; Carter *et al.*, 1945).

(3) Adsorption on charcoal at pH 7·0 followed by elution of water-free charcoal cake with anhydrous methanolic hydrogen chloride, concentration of neutral eluate and precipitation of the streptomycin hydrochloride with acetone (Waksman & Schatz, 1945).

(4) A two-stage process involving solution of the first precipitate in water, adsorption on charcoal, elution with acid-methanol, concentration and precipitation (LePage & Campbell, 1946).

The adaption of the foregoing processes to large-scale manufacturing conditions presented the following difficulties: (*a*) the construction of acid-resistant plant; (*b*) the use of large volumes of methanol, with consequent high cost of production; (*c*) the production of large volumes of methanol-acetone mixtures, presenting difficulties of fractionation; (*d*) the difficulty of recovering small weights of a hygroscopic product from large volumes of solvent.

Bearing in mind these difficulties, a process was developed along the following lines:

(1) Adsorption of the streptomycin from the culture filtrate on 1 % (w/v) activated charcoal at pH 6–8.

(2) Elution of the streptomycin from the charcoal with 1·2 % (v/v) aqueous phosphoric acid at pH 1·0–2·0.

(3) Adsorption of the streptomycin on 3–4 % (w/v) activated charcoal from the neutralized phosphate eluate at pH 7.

(4) Elution of the streptomycin under anhydrous conditions with acidified methanol.

(5) Evaporation of the neutralized methanol eluate to one-eighth of its original volume.

(6) Precipitation of the streptomycin by dilution of the concentrate with acetone, or other precipitant of higher boiling-point, e.g. amyl acetate.

This process has some advantages. For example: (i) In the first stage of the extraction, streptomycin is eluted from the charcoal with dilute aqueous phosphoric acid, enabling stainless steel equipment to be used when volumes are large. (ii) The organic solvent elution stage is delayed until a considerable volume diminution has been effected, decreasing solvent requirements to at least one-tenth of those employed in other processes. (iii) Precipitation of streptomycin is not accomplished until volumes are comparatively small. (iv) The volume of methanol-acetone mixture produced during the process is at a minimum. (v) The use of aqueous phosphoric acid as an eluting agent gives pigment-free eluates and renders unnecessary the introduction of a special decolorizing stage.

METHODS

The method of streptomycin assay was a dilution test using *Escherichia coli* (*Bacterium coli*) as the test organism, and the unit employed was 1 μg. streptomycin base (Brown & Young, 1947). It was necessary to remove methanol by evaporation from concentrated methanol solutions to avoid interference by the bactericidal properties of the alcohol. In practice, samples were evaporated under reduced pressure to 8 % or less of their original volume and diluted back to the initial volume with distilled water. Unsatisfactory results were obtained with certain phosphate eluate samples, and data obtained for extraction efficiencies and potencies up to the phosphate eluate stage are approximations.

The potencies of the final dried products obtained from the pilot plant were also determined by a spectrographic assay method (unpublished) developed by Dr Tudor S. G. Jones of the Wellcome Chemical Research Laboratories (see Table 5).

Total solids were determined by drying in an oven at 70° for 18 hr. Such results agreed satisfactorily with determinations made by drying by sublimation of the water from the frozen state.

LABORATORY-SCALE EXTRACTION

Most of the work on adsorption and elution from charcoal was carried out on laboratory-size Metafilters (Metafiltration Ltd., Hounslow, Middlesex) designed to take a charge of 150 g. charcoal and 115 g. filter-aid. This gave a charcoal bed sufficient for a 15 l. culture filtrate cycle. The beds were washed with water prior to each elution and at the end of each complete and successive cycle.

The extraction process from culture filtrate to the first eluate may be referred to as *stage* 1; and from the first eluate to the final product as *stage* 2. All volumes of washing liquids and eluants used in stage 1 are given as volumes/100 vol. of culture filtrate. For stage 2, all volumes are given as volumes/100 vol. phosphate eluate. Thus a '40 volume' wash means, in stage 1, washing with a volume of liquid 40 % of the volume of culture filtrate taken, and in stage 2, 40 % of the volume of phosphate eluate. The ratio streptomycin to total solids (μg./mg.) is referred to as 'potency'.

After removal of the mycelial felts the culture fluid was clarified by filtration through cloth filters precoated with Hyflo Supercel (Johns Manville Ltd.). The culture filtrate assayed between 200 and 400 μg. streptomycin/ml. on harvesting (potency 6–18). The pH of the culture filtrates ranged between pH 6 and 8.

Stage 1; *First charcoal adsorption*

'Farnell 14' was found to be the most suitable active charcoal for the adsorption of streptomycin from these culture filtrates and allowed elutions to take place with suitable eluants. A level of 1 % (w/v) charcoal to culture filtrate volume appeared optimal for pH 6–8. Pigment adsorption increased, whilst total solid and streptomycin adsorption decreased below pH 6, and at pH 2 almost pigment-free filtrates were obtained without appreciable adsorption of streptomycin. The effect of culture filtrate pH on streptomycin adsorption efficiency is indicated in Table 1.

Table 1. *Effect of* pH *on streptomycin adsorption efficiency*

Original titre of culture filtrate: 148 μg./ml. Adsorption on 'Farnell 14' charcoal: 1 % (w/v).

pH of adsorption	Streptomycin in culture filtrate after adsorption (μg./ml.)	Adsorption efficiency (%)
4	67	53
5	30	80
6	< 11	> 93
7	< 11	> 93
8	< 11	> 93

No differences in efficiency of adsorption of streptomycin were observed as between passing culture filtrates through static charcoal beds, and adsorption on charcoal suspensions. Similarly, static elution appeared as satisfactory as elution by suspension. Static charcoal beds used for successive adsorptions and elutions retained their efficiency, and no appreciable quantity of solid material accumulated in them. One bed could be used for at least seven complete operations. Table 2 indicates the total solid (mg./ml.) recovery between culture filtrate and eluate over seven successive cycles.

Table 2. *The use of one charcoal bed for a series of adsorptions and elutions*

	Cycle							
	1	2	3	4	5	6	7	Mean
Culture filtrate (500 ml.); total solids (mg./ml.)	18·4	18·4	18·4	18·4	18·4	18·5	18·4	—
Spent culture filtrate (500 ml.); total solids (mg./ml.)	—	15·4	15·9	18·4	16·0	15·6	15·4	—
Phosphate eluate (200 ml.); total solids (mg./ml.)	—	5·7	8·8	5·8	6·0	5·8	5·7	—
Washwater (200 ml.); total solids (mg./ml.)	—	2·4	2·6	3·8	2·7	3·0	2·1	—
Total solids of culture filtrate:								
Adsorbed on charcoal (%)	—	16	13	—	13	15	16	15
Recovered in phosphate eluate (%)	—	12	—	13	13	13	12	13

The effect of water washing the charcoal beds after the adsorption of streptomycin was studied. Approximately 30–40 % of the adsorbed solids could be removed without appreciable loss of streptomycin. The progressive change in total-solids content of the effluent when water-washing the charcoal beds is indicated in Fig. 1. A 40 volume water-wash appears to be optimum.

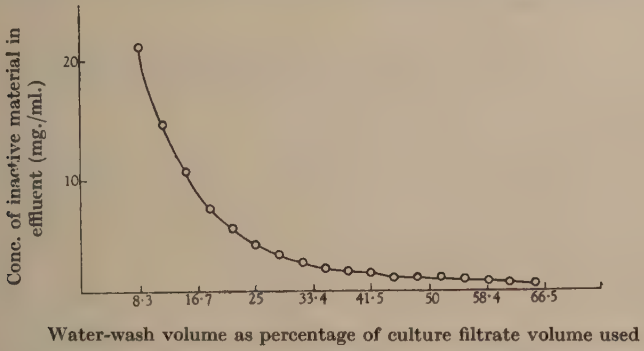

Fig. 1. Relation between water-wash volume and concentration of inactive material in effluent.

Stage 1; *Elution of first charcoal adsorbate*

The progressive change in streptomycin content and pH of the eluate on elution with 1·2 % phosphoric acid is shown in Fig. 2. The first 3–5 volumes of eluate were almost inactive. Elution was complete with 15 volumes. Provided a 40 volume water-wash was introduced between the adsorption and elution, the phosphate eluates were almost colourless. Elution of the water-washed charcoal bed with acid-methanol gave pigmented eluates.

An indication of the order of recovery and volume diminution obtained from culture filtrate to phosphate eluate is given in Table 3. The potency of the phosphate eluate was estimated as 30–60, but the accuracy of the recovery figures is affected by difficulties experienced in assaying streptomycin in the presence of phosphate.

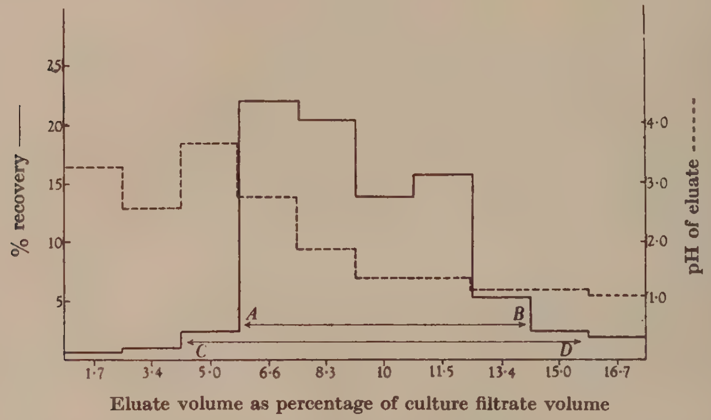

Fig. 2. The progressive change in streptomycin content (continuous line) and pH (broken line) during elution with 1·2 % phosphoric acid. *A–B*, for 10,000 l. culture filtrate to 830 l. eluate, 8·5 % streptomycin loss; *C–D*, for 10,000 l. culture filtrate to 1200 l. eluate, 3·6 % streptomycin loss.

Table 3. *Volume diminution for culture filtrate to phosphate eluate and % recovery*

Culture filtrate volume (1)	15	15	15	15	15	
Phosphate eluate volume (1)	2·5	2·0	2·0	2·0	2·3	
Eluate volume as % filtrate volume	16	13	13	13	15	
Total streptomycin (g.):						
In culture filtrate	1·5	2·27	2·42	2·42	1·43	
In eluate	1·10	1·58	1·46	1·57	1·18	
						Mean
% recovery	73	68	61	65	82	69·8
Culture filtrate: Titre (μg./ml.)	100	151	161	161	95	
Potency (μg./mg.)	—	6·3	7·5	7·0	3·1	

Neutralization of the phosphate eluate with caustic soda gave heavy precipitates of calcium phosphate, calcium ion having been extracted from the charcoal bed during elution at low pH. These precipitates were troublesome to handle, particularly on a manufacturing scale, and entailed an additional filtration. The amount of precipitate could be decreased, but never completely eliminated, by washing the charcoal beds with a large volume of dilute aqueous phosphoric acid prior to the adsorption of streptomycin.

Stage 2; Second charcoal adsorption and elution

The readsorption of streptomycin from the neutralized phosphate eluate was effected, as at the first extraction stage, with Farnell 14 charcoal, and the subsequent elution was by means of acidified methanol. Both operations were carried out with the charcoal suspended in the liquor; 3–4 % (w/v) charcoal was optimal.

Adsorption. After suspension for 30 min. the charcoal was filtered off and the bed washed first with 40 volumes of water and finally with 40 volumes of neutral methanol. The purpose of the neutral methanol wash was to remove some inactive material and to lower the water content of the charcoal bed to a minimum. Failure to remove water from the charcoal resulted in sticky and hydrated precipitates of streptomycin hydrochloride at the precipitation stage.

Elution. This was effected by suspension of the washed charcoal in methanol containing 2 % (v/v) conc. hydrochloric acid (pH 1·5–2·0) with agitation for 30 min. 15 volumes of eluant were used, and while 2 % (v/v) of conc. HCl was usually adequate to bring the suspension to pH 1·5, it was advisable to check the pH after 15 min. stirring, and to add more acid if required. The spent charcoal was removed by filtration and the charcoal bed washed three times on the filter with 5 volumes of acid-methanol. The bulked eluate and washings were adjusted to pH 6 with 80 % (w/v) caustic soda. Calcium phosphate was again precipitated, but the amount of this precipitate also could be decreased by pretreatment of the charcoal with aqueous acid. Recoveries and potencies for the second adsorption and elution are given in Table 4.

Table 4. *Volume diminution phosphate eluate to methanol eluate and % recovery*

				Mean
Phosphate eluate volume (l.)	**2·0**	**2·3**	**2·4**	
Methanol eluate (l.)	**0·38**	**0·44**	**0·40**	
Methanol eluate vol. as % phosphate eluate vol.	**19**	**19**	**17**	
Total streptomycin (g.): In phosphate eluate	**1·57**	**1·18**	**1·05**	
In methanol eluate	**1·06**	**0·81**	**0·66**	
% recovery	**68**	**69**	**62**	**66·3**
Methanol eluate potency (μg./ml.)	**248**	**245**	**—**	

Stage 2; Concentration of second eluate and separation of streptomycin hydrochloride

The neutralized and filtered eluate was concentrated to one-eighth of its original volume by evaporation under reduced pressure at 30–40° and refiltered. Some inactive material, mainly sodium chloride, was thrown out of solution during concentration and the streptomycin potencies increased in consequence. There was no appreciable inactivation of streptomycin during concentration; the pH tended to fall.

White flocculent precipitates of streptomycin hydrochloride were obtained by the addition to the filtered concentrates of five times their volume of commercial acetone. The precipitates were filtered off and dried under reduced pressure over sulphuric acid. Losses of streptomycin in the mother liquor were not more than 1 % of that in the culture filtrate, provided the water content of the methanol-acetone mixture was at a minimum. No detectable increase in potency occurred during precipitation. The overall extraction efficiencies from culture filtrate to precipitate averaged 40 %, and the potency of the precipitate was 200–500.

The solubility of the product in methanol was 10·9 g./100 ml., in ethanol 0·5 g./100 ml., and in methylated spirits 1·1 g./100 ml. The solubility of streptomycin hydrochloride in these solvents will be influenced to some extent by the water content, but it is apparent that, of the three solvents investigated, methanol alone is suitable as an eluting agent. With methanol eluates, care had to be taken to avoid concentration to such a degree that the solubility of streptomycin hydrochloride was exceeded.

The use of acetone as precipitant on a manufacturing scale would be uneconomic owing to the difficulties of fractionating mixtures of methanol and acetone. A search was made for alternative precipitants, of suitable availability, cost, boiling-point, solubility in methanol, water content at saturation, and ease of dehydration. Amyl acetate and monochlorobenzene were the most satisfactory and gave as good streptomycin recoveries as did acetone.

Stability

The stabilities of the crude culture filtrate, the methanol eluate and of aqueous solutions of streptomycin hydrochloride were investigated at various pH values and temperatures. The maximum stability of culture filtrates and aqueous and methanol solutions of streptomycin hydrochloride was in the range pH 3·0–6·0. Aqueous solutions of streptomycin hydrochloride were kept at pH 1·0 and 20° for 173 hr. without loss of activity; aqueous and methanol solutions were kept for 33 days at 20° and pH 7, without any detectable inactivation. Material dried from the frozen state has been kept at 20° for 67 days, without detectable loss of streptomycin content. Aqueous and methanol solutions of streptomycin hydrochloride were heated at 90° for 7 hr. in the range pH 3·0–6·0 without detectable loss of streptomycin content.

PILOT-PLANT EXTRACTION

Filtration and first adsorption. The culture fluid on the plant scale was harvested in batches of 1500 l. and was clarified by filtration through leaf cloth filters precoated with filter-aid; filter-aid was also continuously injected into the flow-line on the feed side of the filter. The temperature of the liquor was decreased to +3° in a brine-cooled heat exchanger. A battery of ten Metafilters, each filter having a bed of 1·5 kg. of Farnell 14 charcoal and 2 kg. Hyflo-Supercel, was used for the adsorption of streptomycin from each 1500 l. batch. The Metafilter beds were pretreated with 1800 l. 1·2 % (v/v) aqueous phosphoric acid, followed by 1800 l. mains water, before being fed with the harvested metabolism fluid. The adsorption of streptomycin was effected at a flow-rate of 4000 l./hr., i.e. 400 l./hr./Metafilter.

First elution. The beds containing the adsorbed streptomycin were washed with 625 l. mains water, and eluted with 250 l. 1·2 % (v/v) aqueous phosphoric acid, the first 50 l. of eluate being run to waste. The eluates were neutralized with 80 % (w/v) caustic soda and insoluble calcium phosphate removed by sedimentation.

Table 5. *Mean stage potencies, and % recoveries for twenty pilot-scale batches*

	Mean potency (μg./mg.)	Total streptomycin (g.)	Stage	Stage recovery (%)	Overall recovery (%)
Culture filtrate*	18·2	5928		—	—
Phosphate eluate	—	3263	Culture filtrate to phosphate eluate	55	55
Methanol eluate	231	2595	Phosphate eluate to methanol eluate	80	44
Methanol concentrate	362	2565	Methanol eluate to methanol concentrate	99	44
Precipitate	422	2099	Methanol concentrate to precipitate	82	36
Freeze-dried product	389 350·5†	1635	Precipitate to freeze-dried product	77	28

* Mean titre: 262 μg./ml. † Spectrographic assay.

Second adsorption. Each 200 l. batch of neutralized and decanted phosphate eluate was agitated with 4 % (w/v) of previously acid-washed Farnell 14 charcoal, for 30 min. The charcoal was collected on Doulton vacuum filters and washed with 100 l. distilled water, followed by 100 l. neutral methanol. This neutral methanol wash was generally used in three fractions, the first 60 % (v/v) methanol-water, the second 98 % (v/v) methanol-water, and the final wash with 100 % methanol.

Acid-methanol elution. The water-free charcoal cake was suspended and agitated in 30 l. methanol, containing 2 % (v/v) conc. HCl. Elution was effected in 30 min. The spent charcoal was recovered on a Doulton filter and the charcoal bed washed with three 10 l. washes of acid-methanol.

Concentration. The bulked eluate and washings were adjusted to pH 5–6 with 80 % (w/v) caustic soda, and concentrated to one-eighth volume in a climbing film evaporator. The methanol concentrate, of 7–8 l., was filtered on a Buchner filter and the filtrate poured into 40 l. acetone. The precipitated streptomycin hydrochloride was filtered off, dried under reduced pressure, dissolved in pyrogen-free distilled water, Seitz-filtered, and freeze dried in phials, having approximately 160,000 μg./phial. Stage and overall recoveries and potencies are given in Table 5. The overall and stage recoveries are calculated from the mean of twenty batches. The crude filtrate titres and final potencies were appreciably higher than the corresponding laboratory figures. The overall recovery from culture filtrate to freeze-dried product was lower than that obtained in the laboratory, but stage recoveries on the plant approximately paralleled those obtained in laboratory extractions. There was a significant increase in potencies during the concentration of methanol eluates.

We are indebted to Dr Tudor S. G. Jones for the spectrographic assay determinations of dried products, and Dr G. C. Ainsworth and Dr Tudor S. G. Jones for helpful criticism and advice. The pilot-scale production was carried out in the penicillin extraction plant designed by Dr C. G. Pope and Dr W. B. Hawes, and modified by us for the extraction of streptomycin.

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A Dilution Method for the Assay of Streptomycin

By ANNIE M. BROWN and P. A. YOUNG

The Wellcome Physiological Research Laboratories, Beckenham, Kent

SUMMARY: A dilution method for the assay of streptomycin is described using a digest nutrient broth as the test medium and a certain strain of *Escherichia coli* (*Bacterium coli*) as the test organism. The inhibition end-point is estimated turbidimetrically by comparison with that in a standard solution of streptomycin base. The accuracy of the method when employed as described is of the order of ±15%. It is necessary to control the dilution of the test medium, the temperature of incubation of the tests, the size of the inoculum and hydrogen-ion concentration in order to obtain consistent results. With dilution of the medium the sensitivity of the test is increased while its accuracy is decreased. The use of an inoculum of constant size is important, and an incubation temperature of 28° is found to be more satisfactory than 37°. Medium with a hydrogen-ion concentration of pH 7·6 affords greater sensitivity than that with a lower pH value.

During studies of streptomycin production by *Streptomyces griseus* (see Ainsworth, Brown, Marsden, Smith & Spilsbury, 1947; Woodthorpe & Ireland, 1947) it was necessary to assay the activity of the culture fluids. Several methods for streptomycin assay are noted by Waksman & Schatz (1945), but a dilution test similar to that described by Pope & Stevens (1946) for penicillin was favoured for routine use. The method finally adopted, which gives little trouble providing constant conditions are maintained, is described below.

The unit of streptomycin. American workers have used the S unit of Waksman (1945) which was defined as that amount of material which will inhibit the growth of a standard strain of *Escherichia coli* (*Bacterium coli*) in 1 ml. of nutrient broth or other suitable medium. This corresponds to the original *Esch. coli* unit and was said to be equivalent to 1 μg. of crystalline streptomycin. It has, however, since been stated that pure streptomycin base contains approximately 800 units/mg. (Molitor, Graessle, Kuna, Mushett & Silber, 1946). For this reason it is considered less ambiguous to express activities as μg. of streptomycin base/ml. or /mg. and so avoid the use of unstandardized units.

The standard used. Three samples of streptomycin sulphate were received from America, two from Merck and Co. Inc., and one from Chas Pfizer and Co. Inc., the former labelled with the weight of streptomycin base/phial, and the latter with its purity specified. It was deduced from the weight of material in the phials received that the first two samples each contained 500 μg. of streptomycin base/mg. This agreed, within the limits of error of the test, with the stated streptomycin content of the third sample. An independent spectrographic method of assay developed by Dr Tudor Jones of the Wellcome Chemical Research Laboratories (unpublished), gave essentially the same titres for the three American samples as did the biological assay. The Merck sample was considered as standard for pharmacological reasons (Madigan, Swift & Brownlee, 1947).

Test organism. The organism used for this test was *Esch. coli* strain CN 636, available in the dried state at the Wellcome Physiological Research Laboratories, Beckenham. This organism is easy to control and its non-pathogenicity is in its favour.

Test medium. The influence of various substances such as glucose, thiol compounds and ketone reagents, and of hydrogen-ion concentration on the activity of streptomycin has been noted by a number of workers (Denkelwater, Cook & Tishler, 1945; Geiger, Green & Waksman, 1946; Donovick & Rake, 1946; Abraham & Duthie, 1946; Bondi, Dietz & Spaulding, 1946; Wolinsky & Steenken, 1946; Van Dolah & Christenson, 1947). In experiments described below the action of certain of these compounds could be correlated with the effect on hydrogen-ion concentration, but for others, particularly thiol compounds and ketone reagents, the nature of the effect was obscure. The sensitivity to these compounds and to the concentration of peptone used, as described by Donovick & Rake (1946), made a control of the test medium very necessary (cf. Hobby, Lenert & Hyman, 1946). A nutrient broth which could readily be produced in large batches by the method detailed below was found to be very suitable.

DETAILS OF THE ASSAY METHOD

Preparation of the nutrient broth. Minced horseflesh (20 lb.) was mixed with tap water (9 l.) and heated to 60°. The pH was then adjusted to 5·2–5·6 with HCl and 30 g. of papain was added in 10 g. quantities hourly. During digestion the temperature was kept between 58 and 62°. After 4 hr. the pH was adjusted to 5·0–5·5 with HCl and the product boiled and filtered. The filtrate was reheated to 90° and then allowed to stand overnight, after which it was filtered and stored.

A sufficient quantity of this papain digest was diluted with tap water to give a total N content of 1·6 g./l. Sodium chloride was added to give a final concentration of 0·5%, allowance being made for that already present in the medium. A mixture was then made containing the proportions 1 l. of this diluted digest and 1 lb. of minced horse muscle. This mixture was heated to 65°, maintained at that temperature for 20 min., then boiled for 30 min. The meat particles were allowed to settle and the supernatant liquid decanted. Liquor remaining in the meat was squeezed out in a press, and the whole volume of liquor was collected and made up with distilled water to the original volume of papain digest used. The broth was adjusted to pH 7·6 with NaOH, boiled, and filtered through paper or cloth precoated with Hyflo Supercel (Johns Manville Ltd.) according to the size of the batch.

When preparing medium for the assay several bottles of nutrient broth prepared as above were bulked and the pH checked. The medium was then distributed in volumes of 5·0 ml. by an automatic measurer into 4 × 1 in. tubes. These were autoclaved for 10 min. at 10 lb./sq.in., a procedure which did not affect the volume. The pH of the broth after autoclaving was also noted, as any variation of more than 0·1 pH unit had an appreciable effect on the end-point dilution of the streptomycin standard.

Maintenance of culture and preparation of inoculum. The *Esch. coli* strain was maintained on nutrient agar slopes. In order to diminish the frequency of subculture, each week six slopes were inoculated from the parent slope and after 24 hr. growth at 37° were stored in a cold room. Each day of the week following subcultures into nutrient broth were made from one of these slopes, incubated for 17 hr. and held at 4° until required for use. A satisfactory inoculum was obtained by using 0·2 or 0·3 ml. of a 10^{-6} dilution of such a 17 hr. culture. Inoculation by means of a wire was unsatisfactory because the organism is very sensitive to heat and inoculation tended to be patchy.

Preparation of standard streptomycin solution. A sufficient volume of a solution of the standard streptomycin sulphate containing 200 μg./ml. was dispensed in phosphate buffer (0·1 % Na_2HPO_4 adjusted to pH 7 with phosphoric acid) to last at least a week. No deterioration of this solution has been detected when stored in the cold room. Before addition to the series of tubes for assay this solution was diluted 10 or 15 times with nutrient broth. A small amount of chloroform was added to the original phosphate buffer to preserve sterility, and all other dilutions were made with sterile precautions.

Determination of assay end-point. The weight of streptomycin base producing inhibition in growth of *Esch. coli* was determined in the following manner. Standard streptomycin solution was added to a series of 5 ml. amounts of broth in the following volumes (ml.): 1·4, 1·3, 1·2, 1·1, 1·0, 0·9, 0·8, 0·7, 0·65, giving a '10 % range' with a dilution factor of 1·07–1·12. Each tube was inoculated with 0·3 ml. of a 10^{-6} dilution of 17 hr. culture of *Esch. coli* in nutrient broth, shaken vigorously, and incubated for about 16 hr. at 28°. The tube in which only a trace of growth appeared was taken as the end-point.

As a general rule there was rapid transition from no growth to positive growth, and the end-point tube was not difficult to locate. The amount of streptomycin present in the end-point tube was then calculated. For example, when this tube contained 0·9 ml. of the 1/10 dilution of the standard 200 μg./ml. streptomycin solution, the total volume being 6·2 ml. (5·0 ml. broth + 0·9 ml. streptomycin dilution + 0·3 ml. inoculum), the amount of pure streptomycin base/ml. is given by

$$\frac{200}{10} \times \frac{0{\cdot}9}{6{\cdot}2} = 2{\cdot}90\,\mu\text{g. pure base/ml.}$$

This end-point varied slightly from day to day, and it was necessary therefore always to include two standard ranges among each day's tests.

THE METHOD OF ASSAY

Samples for assay were saturated with chloroform to inhibit the growth of any chance contamination. As the least possible dilution of a sample was 1/3·5 and was usually much greater, there was no danger of inhibition of growth by the chloroform alone, a saturated solution of which does not inhibit growth at greater dilutions than 1/2.

When testing samples of unknown value ranges covering volumes from 2·0 to

0·5 ml. of the sample at two levels of dilution were generally used, e.g. 5 ml. tubes of broth with the addition of:

Undiluted sample (ml.): 2·0 1·3 0·8 0·5; dilution factor 1·5
Sample diluted 1:3 (ml.): 2·0 1·3 0·8 0·5; dilution factor 1·5.

If considerable amounts of streptomycin were suspected larger dilutions of the sample were made. In all tests the final ranges were similar to those shown in the paper by Pope & Stevens (1946), with dilution factors 1·20–1·10. All dilutions were made with sterile precautions in nutrient broth at pH 7·6. This was essential owing to the effect of the dilution of the medium on the activity of the streptomycin (see Donovick & Rake, 1946; and below). The tests were inoculated in the same way as the standard dilutions and incubated for 16 hr. at 28°.

In order to facilitate calculation of the potency of samples, tables were calculated for all possible variations of the standard end-point. A small portion of one of these tables is shown in Table 1.

Table 1. *Part of table giving the relation of dilution end-points of standard and sample*

End-point of standard (20 µg./ml.) (ml.)	Streptomycin base equivalent (µg.)	Streptomycin base equivalents for dilutions of samples giving end-points at: Volume of dilution added (ml.)											
		2·0	1·8	1·6	1·4	1·3	1·2	1·1	1·0	0·9	0·8	0·7	0·65
		Equivalents of streptomycin base (µg.)											
1·1	3·54	12·5	13·5	14·5	16·0	17·0	18·5	19·5	21·0	23·0	25·5	29·0	31·0
1·0	3·22	11·5	12·0	13·5	14·5	15·5	16·5	18·0	19·5	21·0	23·5	26·0	28·0
0·9	2·90	10·0	11·0	12·0	13·0	14·0	15·0	16·0	17·5	19·0	21·0	23·5	25·0

If the standard end-point was on 1·0, and the sample dilution end-point on 1·4, then from the table the sample dilution contained 15·0 µg. of streptomycin base/ml. This multiplied by the dilution of the sample gave its potency.

The accuracy of the potency determinations can be seen from Table 2. No special choice of workers was made when assaying these samples. From the standard deviation of a single test it is clear that the potency of a sample can be determined as described above to about ±15%, which is satisfactory for a routine assay of this nature.

Table 2. *The accuracy of the streptomycin assay*

Series	No. tests	Potency (µg./ml.)	Standard deviation of single test	Coefficient of variation (%)
A	11	172	±36·7	±21·3
B	12	318·8	±46·4	±14·5
C	19	293·5	±41·5	±14·2
D	16	167·5	±19·2	±11·5
E	20	156·3	±13·4	± 8·6

Comments on the assay method

Concentration of broth. It was found that dilution of the test medium resulted in a higher relative activity of the streptomycin. Thus a fourfold dilution of broth increased the end-point dilution of samples approximately fifty-fold, growth remaining satisfactory and easily detectable in such a weak medium. This increase in the sensitivity of the test was an advantage in the assay of low titre samples. A series of results for a sample of crude streptomycin assayed on different days in nutrient broth of pH 7·0 are given in Table 3. The results for undiluted broth are each the mean of about 30, while those for the weaker medium are each the mean of about four results.

Table 3. *Variation of end-point dilution of a crude streptomycin sample with dilution of test broth*

Showing dilution of the sample required to produce end-point growth in each of six replicate tests using two strengths of nutrient broth.

Test broth	End-point dilution of sample					
Nutrient broth: Undiluted	1/ 41	1/ 40	1/ 31	1/ 30	1/ 31	1/ 22
Diluted 1/4	1/1100	1/2100	1/2900	1/620	1/2400	1/910

Temperature of incubation. During the first few weeks of streptomycin assay when tests were incubated at 37 and not 28° (see Geiger *et al.*, 1946) it was noticed that the growth of *Esch. coli* was irregular at the inhibition end of the test. Shaking the tubes vigorously after inoculation diminished the number of tubes growing unevenly, and incubation at 28° was much more satisfactory than at 37°. This latter point was proved by counting the number of tubes each day where *Esch. coli* had multiplied freely in dilutions of antibiotic less than those showing inhibition both in tests incubated at 37 and at 28°. A statistical analysis of these 'odd tube' counts was made using Poisson's series. For 1642 tests the mean number of tubes out of order at 37° was 11·16 % and for 1880 tests at 28° it was 3·03 %. The difference between these two means is highly significant ($P=0{\cdot}0078$).

Quantity of inoculum. Some of the early assays were carried out in modified penicillin assay broth (Pope & Stevens, 1946) and in general the inoculum used for tests in papain-digest medium was 0·2 ml. of a 10^{-6} dilution of broth culture. Variation of this volume gave results which suggested that the end-point dilution is roughly inversely proportional to the quantity of inoculum/ml. of test broth (see Table 4) and indicated that care must be taken to use a constant inoculum.

Table 4. *Variation of end-point dilution with size of inoculum*

The table relates the dilution of sample required for end-point growth, with volume of inoculum (10^{-6} dilution of 17 hr. broth culture of *Esch. coli*) at two concentrations of medium. The broth used was a papain-digest of beef muscle, pH 9·0.

Test broth dilution	Volume of inoculum (ml.)	End-point dilution
1/4	0·2	1/257
	0·4	1/143
1/16	0·2	1/47000
	0·8	1/8200

The effect on assay values of different pH *values of the test broth and of the addition of oxidizing and reducing agents*

The effect of pH. Reference has already been made to published work on the effect of various agents added to the test broth on the streptomycin assay values. The discovery that vigorous shaking often gave more consistent results suggested that oxidizing and reducing agents might have significant effects on the action of streptomycin in the assay. As some of these agents also had an effect on the pH of the test broth the effect of variation of the pH on the end-point concentration of streptomycin base was first investigated (see Table 5). It was found during this experiment that the pH of the broth used for the routine tests fell from pH 7·6 to 7·4 on re-autoclaving. The variation in activity from pH 7·2 to 7·4 and from pH 7·4 to 7·6 was rather more than 20 %. This means that for each decrease of 0·1 unit of pH between 7·6 and 7·2 the streptomycin activity was decreased by *c.* 10 %. Wollinsky & Steenken (1946) showed that the greatest diminution in activity occurred between pH 6·6 and 5·9.

Table 5. *Variation of amount of streptomycin base at the end-point in relation to the* pH *of the test broth*

Experiment	pH of broth	Average weight of streptomycin base at end-point (μg.)	Percentage variation in activity
A (16 determinations)	7·2	2·73	−20 to +24
B (16 determinations)	7·4	2·19	−22 to +28
C (7 determinations)	7·6	1·71	

The effect of certain oxidizing and reducing agents. In the determination of the effect of oxidizing or reducing agents a concentration of substance was used such that growth of the organism in its presence was comparable to that in broth alone, and in all experiments controls of growth were included. The limit of growth was measured as the greatest dilution at which opalescence was apparent in a ten-fold dilution series. The concentration of the material was also varied by tenfold, starting at a 1/5 or 1/10 dilution. Diverse substances were chosen so that no common chemical group would be responsible for the oxidation or reduction. The concentration of each of the agents which inhibited growth in broth alone, inhibited growth in the presence of streptomycin, with the exception of hydrogen peroxide ('20 vol.' solution) however, which inhibited growth in broth alone at a dilution of 10^{-3} but not in the presence of streptomycin.

The results in Table 6 are expressed as the percentage change in the number of μg. streptomycin base estimated as being present at the end-point of the experimental test as compared with those in the control routine test.

The changes in streptomycin activity observed when using the reducing agents was partly due to change in pH. Hydroxylamine hydrochloride and thiolacetic acid show very little effect on the streptomycin titre (cf. Cavallito, 1946). The action of sodium dithionite was different, only about a third of the total effect being due to pH change.

Table 6. *Variation in the activity of streptomycin in the presence of certain oxidizing and reducing agents at the maximum concentration having no effect on the growth of* Esch. coli

Reagent	Final dilution in test broth	Change in pH of broth	% change in streptomycin base present at end-point: Crude filtrates	% change in streptomycin base present at end-point: Purified streptomycin
Oxidizing agents:				
Iodine	10^{-4}	None	+ 3·8	− 0·6
K permanganate	10^{-4}	None	+15·3	+16·0
Hydrogen peroxide (20 vol.)	10^{-4}	None	+ 4·5	+ 3·6
Reducing agents:				
Sodium dithionite ('hydrosulphite')	10^{-3}	−0·2	+75	+72·4
Hydroxylamine HCl	2×10^{-5}	−0·1	+19·6	+18·5
Thiolacetic acid	10^{-4}	−0·1	+22	+12

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Studies on Soil Acrasieae

2. The Active Life of Species of *Dictyostelium* in Soil and the Influence thereon of Soil Moisture and Bacterial Food

By B. N. SINGH

Department of Soil Microbiology, Rothamsted Experimental Station, Harpenden, Herts

SUMMARY: When spores of *Dictyostelium mucoroides* or *D. giganteum* were added to sterilized soil containing a pure culture of an edible bacterium, the resulting myxamoebae actively destroyed the bacteria in the soil. When spores of *D. mucoroides* were added to the centre of a Petri dish of sterilized soil containing bacterial food no fruiting bodies were formed at a moisture content of 15 % or less. At 19 % moisture fruiting bodies were formed at the centre only. At 33 % moisture the central area over which fruiting bodies appeared steadily increased in size until it covered the whole soil surface. Species of *Dictyostelium* can also pass through the life cycle in fresh unsterilized soil.

The nature of the bacterial food supply affects the growth of species of *Dictyostelium* in soil as measured by fruiting body formation. Normal fructification occurs on soil containing certain bacterial strains which induce abnormal fructification on agar.

It has been shown (Singh, 1946*c*, 1947) that species of *Dictyostelium* are frequently present in arable soils in Great Britain. Their common occurrence in soils of the classical plots at Rothamsted, which have been unmanured or treated only with artificial fertilizers for more than 100 years, suggests that they are true soil inhabitants. The presence of *Dictyostelium* species in dung which has been lying on the ground for some time indicates that the organisms find suitable bacterial food in these substrates for their development. Among the large variety of bacterial species offered as food to two species of *Dictyostelium* on non-nutrient agar substrate (Singh, 1947), some were completely eaten, either readily or slowly, whereas others were only partly eaten and the rest were inedible. In soil economy the importance of Acrasieae depends, first, on whether the myxamoebae can lead an active and trophic existence in soil and, secondly, on whether they are present in sufficient numbers to diminish the bacterial population. This paper deals principally with the first question.

EXPERIMENTAL

Material. Two species, *Dictyostelium mucoroides* (Brefeld, 1869) and *D. giganteum* (Singh, 1947), were used, which had been isolated from soils and compost heaps by the method of Singh (1947).

Diminution of bacterial numbers by the myxamoebae of Dictyostelium mucoroides *and* D. giganteum *in sterilized soil*

Soil from the plot on Barnfield at Rothamsted, annually manured with 14 tons of farmyard manure per acre, was air dried and passed through a sieve of 3 mm. mesh. It was placed in 300 g. lots in large 7·5 in. diameter Petri dishes and autoclaved for 2 hr. at 15 lb./sq.in. Tests showed that the soil was sterile. The inocula used were: (*a*) bacteria, (*b*) bacteria + spores of *D. mucoroides*, (*c*) bacteria + spores of *D. giganteum*.

A common soil bacterium (strain 4002) which is readily and completely eaten by the myxamoebae and on which normal fruiting bodies of *Dictyostelium* spp. are produced, was selected for this experiment. One or two loopfuls of a 2–3-day culture of bacterium 4002 on nutrient agar were spread in a thin layer on non-nutrient agar (1·5 % agar in 0·5 % NaCl; pH 6·5–6·8) in Petri dishes. A spore mass, or sorus, raised well above the surface of its culture medium, was gently punctured by a fine sterilized needle and the spores added to the bacterial culture on the non-nutrient agar. When fruiting bodies were formed, within 3–4 days, the spores were inoculated on to freshly prepared bacterial cultures. This process was repeated until cultures of *D. mucoroides* and *D. giganteum* growing on pure cultures of bacterium 4002 were obtained. For inoculation of the soil plates, growth from 2–3-day cultures of bacterium 4002 on nutrient agar slopes was gently scraped off, suspended in 0·5 % NaCl and the number of bacteria/ml. determined by a haemocytometer count. Approximately the same number of bacteria was inoculated into each of the soil samples contained in the large Petri dishes. During inoculation the soils were shaken to distribute the bacteria as uniformly as possible. Large numbers of spores of *D. mucoroides* and *D. giganteum* produced on bacterium 4002 were then suspended in 0·5 % NaCl and the number/ml. determined in a haemocytometer. Approximately the same number of spores, 64,000/g. soil, was added to each soil. The moisture content of the soils was brought to 33 % (on dry-weight basis) by adding sterilized tap water. Immediately after the double inoculation counts of the bacteria were made by plating from each of the soils. The number of viable bacteria contained in each soil sample was approximately the same. The soils were then incubated at 19–20°.

The counts of viable bacteria were made at intervals. From each Petri dish ten random samples were taken by means of a sterilized cork borer; they were thoroughly mixed and 10 g. of the mixture used to make the necessary dilutions. The results are shown in Fig. 1. In the control soil the highest bacterial count was obtained in 10 days. Over the whole period of the experiment the rise in bacterial numbers was much smaller in the soils inoculated with *Dictyostelium* spp. It was most marked in the first sample, after 3 days' incubation. This was probably due to the fact that after the myxamoebae emerged from the spores, they multiplied rapidly for a few days before giving pseudoplasmodia and fruiting bodies. It is during this intensive reproduction stage that large numbers of bacteria are consumed by the myxamoebae. Once the latter begin to aggregate to pseudoplasmodia, the myxamoebae cease feeding. Within 3–5 days fruiting bodies were seen over the soil surface in large numbers. It is thus natural that when most of the myxamoebae have given rise to pseudoplasmodia the consumption of bacteria should be much diminished.

The destruction of bacteria by *D. mucoroides*, isolated from soil, was more marked than that by *D. giganteum*, which was isolated from an actively decomposing compost heap of straw and sludge.

The spread of myxamoebae of Dictyostelium *in sterilized soil and the formation of fruiting bodies at different moisture contents*

Myxamoebae of *Dictyostelium* are able to feed upon dead bacteria and possibly also upon dead cells of other micro-organisms present in the sterilized soil. When the spores or myxamoebae were inoculated into sterilized soil to which no viable bacteria had been added, some fruiting bodies were formed in soil at moisture contents between 20 and 40 %. The formation of fruiting bodies at different moisture contents of the soil is described below.

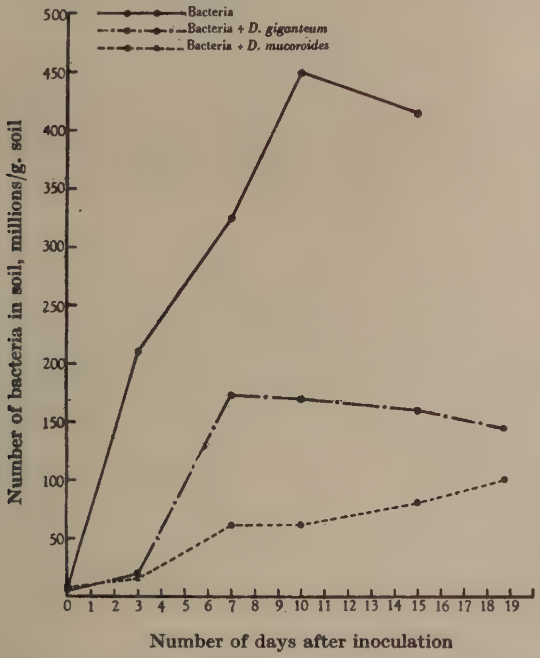


Fig. 1. The growth of bacterium 4002 in sterilized soil, alone and in the presence of *Dictyostelium* spp.

Two hundred and fifty g. portions of air-dried sifted soil were put into large Petri dishes (7·6 in. diameter) and sterilized. Their moisture contents were adjusted roughly to 8, 10, 15, 19, 25 and 33 % by dry weight, and a young culture of bacterium 4002 suspended in 0·5 % NaCl added. Approximately the same number of bacteria/g. soil was inoculated into each of the soils. A cylinder of soil (0·5 in. diameter) was removed from the centre of each Petri dish by a cork-borer, and the space thus made was filled with air-dried and sterilized soil. Upon this was placed one drop of a thick suspension of spores of *D. mucoroides* in 0·5 % NaCl. The small amount of dry soil absorbed the drop of water and thus prevented a local accumulation of moisture which might have led to the water currents carrying the spores outwards. Lids of small 1 in.

Petri dishes were placed on the top of this soil over the site of the *Dictyostelium* seeding to stop the spread through the air of spores from fruiting bodies produced at the centre. The plates were incubated at 20–21°. Observations on the development of fruiting bodies on the soil surface are recorded in Table 1.

Table 1. *The approximately circular area occupied by fruiting bodies when spores of* Dictyostelium mucoroides *were inoculated centrally in 7·5 in. Petri dishes of sterilized soil of different moisture content*

Moisture content of soil (%)	Days after inoculation 3	4	5	6	8	13
	Area occupied by fruiting bodies (sq.in.)					
8	0	—	—	—	—	0
10	0	—	—	—	—	0
15	0	—	—	—	—	0
19	0	—	—	—	0·3	0·3
25	0	—	—	0·5	—	3·1
33	0·8	3·1	12·5	28·3	—	176

No fruiting bodies were formed in 13 days in soils with 8, 10 and 15% moisture; at 19% moisture some were formed on the 8th day at the place of inoculation, but they remained confined to a small area in the centre of the Petri dish. Fruiting bodies were produced on the 6th day at 25% moisture and were visible up to a distance of an inch from the site of inoculation on the 13th day. At 33% moisture, fruiting bodies were formed within about ½ in. of the centre of the Petri dish on the 3rd day, and thereafter the area over which they appeared increased steadily until it covered the Petri dish after 7 days. On the 8th day large numbers of fruiting bodies were seen over the whole soil surface. It is interesting to note that by increasing the moisture content of the soil the fruiting bodies not only appeared much earlier but their spread was also much quicker. This suggests that spreading of these *Dictyostelium* spp. is due to migration of myxamoebae through the soil moisture. On the 4th day of the growth at 33% moisture fruiting bodies were seen inside the area enclosed by the lid of the small Petri dish, as well as outside it. It seems probable that in nature Acrasieae spread both by the dispersal of the spores and by the migration of the myxamoebae. When the soils of lower moisture content, in which no fruiting bodies had been formed, were brought to 35–40% moisture, large numbers of fruiting bodies were formed throughout the soil within 7–10 days. The spread of fruiting bodies and their formation was unchanged when the moisture content was raised from 33 to 40%. It is interesting that no fruiting bodies were seen, even at the centre, on soil with less than 15% moisture. It appears that a certain amount of moisture is necessary for the germination of the spores or for the aggregation of the myxamoebae to form pseudoplasmodia.

In the next experiment the myxamoebae of *D. mucoroides* were substituted for the spores and a drop of a thick suspension of the myxamoebae in 0·5% NaCl was inoculated in the centre of the Petri dish (Table 2). At 10% moisture no fruiting bodies were formed; at 15% a few were seen at the place of inoculation on the 3rd day, remaining confined to a small area up to 19 days. At 20 % moisture fruiting bodies appeared on the 3rd day, as with 15 % moisture, but they spread ½ in. within 19 days. The appearance of the fruiting bodies at 25 and 30 % moisture took place on the 3rd day, but their spread was much more rapid at 30 % than at 25 % moisture. At 30 % moisture fruiting bodies were seen throughout the soil surface within 10–12 days. When myxamoebae were inoculated into soil the fruiting bodies appeared a few days earlier at 20 and 25 % moisture than they did when spores were used as inoculum. This suggests that at these moisture levels it takes some time for the spores to germinate and give rise to myxamoebae. At 33 % moisture, however, fruiting bodies made their appearance on the 3rd day when either spores or myxamoebae were used as inoculum. It seems that this higher moisture content is required for the rapid germination of the spores to give rise to myxamoebae. The soil of 10 % moisture, where no fruiting bodies had been formed, was brought to 40 % moisture at the end of 17 days; large numbers of fruiting bodies were then formed throughout the soil within the next 8–10 days.

Table 2. *The approximately circular area occupied by fruiting bodies when myxamoebae of* Dictyostelium mucoroides *were inoculated centrally in* 7·5 *in. Petri dishes of sterilized soil of different moisture content*

	Days after inoculation				
Moisture content of soil (%)	**2**	**3**	**6**	**7**	**19**
	Area occupied by fruiting bodies (sq.in.)				
10	0	—	—	—	0
15	0	0·3	0·5	—	0·5
20	0	0·3	—	—	0·8
25	0	0·5	—	0·8	3·1
30	0	0·5	—	—	176

Inoculation of spores and myxamoebae into natural soil

Rothamsted Barnfield farmyard manured soil was air dried and passed through a sieve of 3 mm. mesh. Fifty g. portions of this soil were put in each Petri dish. Some of these dishes were inoculated with a thick suspension of the spores, some with the myxamoebae of *D. mucoroides* while the others were left as control. The moisture content of the soils ranged between 30 and 40 %. The plates were incubated at 20–21°. Within 7–15 days a few fruiting bodies were formed in some of the soils which received spores or myxamoebae, but no fruiting bodies could be seen in the control soil.

Kubiena and his colleagues (see Kubiena, 1938) in their extensive studies in micropedology by direct observation of various soils have given excellent photographs of stalks and sori which from their appearance are almost certainly those of species of *Dictyostelium*, although they identified them as the fruiting bodies of fungi. The identification of these bodies as Acrasieae is supported by the fact that they could not grow them on media suitable for fungi even after repeated trials (cf. Pl. 1 with the photographs of Kubiena, 1938).

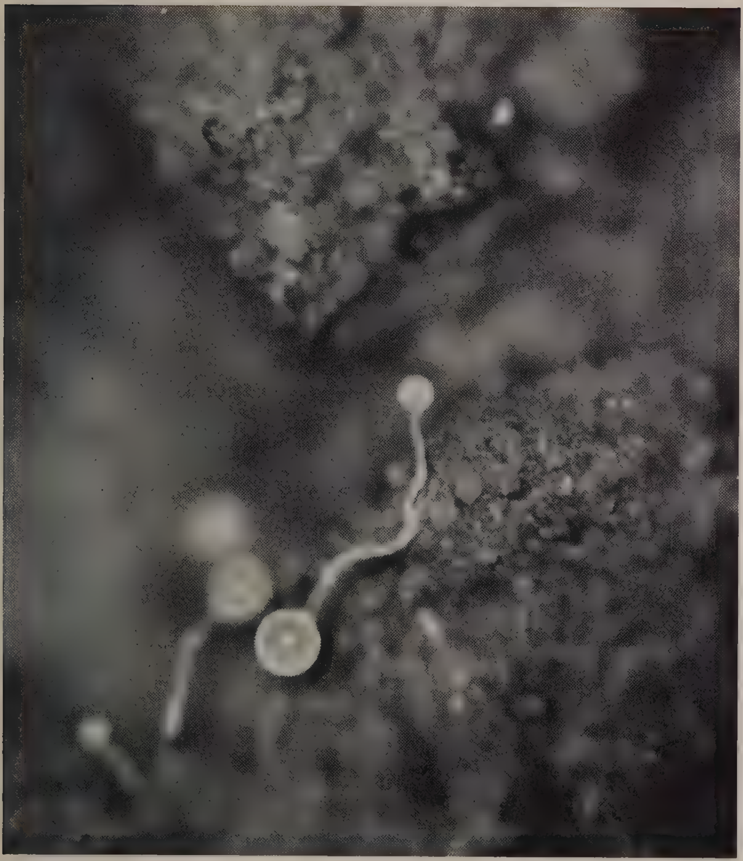
Production of fruiting bodies of Dictyostelium mucoroides *in sterilized soil with the addition of different bacterial strains*

Forty g. portions of Rothamsted Barnfield farmyard manured soil were put into Petri dishes, sterilized, and each inoculated with approximately the same number of bacteria from 2- to 3-day nutrient agar cultures of five strains of common (4000, 4002, 4003, 4022 and 4039) and four strains of rare (2881C, 2650, 4087 and 6699) soil bacteria. The inoculum of the spores of *D. mucoroides* was approximately 20,000/g. soil. A few dishes were inoculated with the spores only. The methods used were those described above. The moisture content of the soil was 33% after sterilization; the plates were incubated at 20–21°. The common and rare strains of bacteria selected were known to multiply well and attain high numbers in sterilized soil. A rough quantitative estimation of the fruiting bodies was made at the end of 10 days (Table 3). Some fruiting bodies were produced from the spores of *D. mucoroides* in the absence of the added bacteria. Large numbers were produced on six of the bacterial strains; and on the other three and in the control soil only very few. Thus the quality of the bacterial flora affected the development of the *Dictyostelium* spp. in soil as reflected in the number of fruiting bodies produced. The effect of these bacterial strains on the fruiting of these species of *Dictyostelium* on non-nutrient agar has already been described (Singh, 1947). Strains 4000, 4002 and 4003 were completely consumed by the myxamoebae on non-nutrient agar and induced abundant and normal fruiting. They also induced numerous fruiting bodies in soil. Strain 4022 was only partly eaten on agar though it induced normal fruiting bodies; it induced very little fruiting in soil. Strains 2881C and 4087 induced abnormal fruiting on agar and very few fruiting bodies in soil. But strains 4039, 2650 and 6699 although they induced abnormal fruiting on agar yet enabled large numbers of normal fruiting bodies to be produced in soil.

Table 3. *The production of fruiting bodies (FB) of* Dictyostelium mucoroides *on sterile soil or non-nutrient agar in presence of different strains of bacteria*

The different strains of bacteria were inoculated into sterilized soil or spread over the non-nutrient agar, and spores of *D. mucoroides* added.

Identification nos. of bacterial strains used	Sterilized soil	Non-nutrient agar	
	Relative numbers of fruiting bodies formed	Characters of fruiting bodies	Edibility of bacteria by the myxamoebae
4000	Large	Normal	Complete
4002	Large	Normal	Complete
4003	Large	Normal	Complete
4022	Very small	Normal	Partial
2881 C	Very small	Abnormal	Partial
4087	Very small	Abnormal	Complete
4039	Large	Abnormal	Partial
2650	Large	Abnormal	Complete
6699	Large	Abnormal	Partial
Control	Very small	—	—



Fruiting bodies of *Dictyostelium mucoroides* growing in sterilized soil inoculated with soil bacterium 4002 (×20).

B. N. SINGH—STUDIES ON SOIL ACRASIEAE. PLATE 1

DISCUSSION

These results show that the myxamoebae of *Dictyostelium mucoroides* and *D. giganteum* are able to multiply and produce pseudoplasmodia and fruiting bodies in soil, whether sterilized or not, containing a suitable bacterial food supply. They also show that for certain strains of bacteria, differential feeding effects occur in soil as well as on agar. The direct evidence that the amoebae of the Acrasieae lead an active life in soil lends support to the indirect evidence derived from dilution counts that the true amoebae are also active inhabitants, since the two types of amoebae are identical in their feeding habit and general mode of life.

It is interesting that the rate of migration of the myxamoebae through soil as estimated from the increasing area over which fruiting bodies appeared was approximately 1 in./24 hr. at suitable water contents. This corresponds well with that found by Thornton & Gangulee (1926) for the spread of *Rhizobium* spp. in sterilized soil.

This work was made possible by a grant from the Agricultural Research Council, to whom the author's thanks are due. It is a pleasure to express my sincere thanks to Dr H. G. Thornton, F.R.S., and Miss L. M. Crump for their interest in this work.

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Spray Inoculation of Plates in the Detection of Antagonistic Micro-organisms

By A. WILSKA

The Wihuri Institute, Helsinki

SUMMARY: By means of a simple atomizer the surface of agar media may be sprayed with a suspension of micro-organisms, so that an even growth is obtained. The method is suitable for the seeding of plates for the assay of antimicrobial substances. It is particularly valuable for the detection of microbial antagonisms, for a test organism may be seeded on to established plate cultures of other organisms, without disturbing the bacterial colonies already present on the agar surface.

The discovery of penicillin has in recent years stimulated the search for other antibiotics. A commonly used method for discovering antagonistic micro-organisms is to distribute a mixed culture from soil, manure, etc., on to the surface of a plate seeded with a suitable test organism. After incubation, a clear zone free from the test bacterium indicates the presence of an antagonist. The drawbacks of this technique are, first, that the potential antagonists, especially if they develop slowly, may be choked by the growth of the test organism, and secondly, a non-lytic antibiotic may leave no visible record of its action. The obvious remedy is to allow the organisms from the soil or other suspension to develop for some time before seeding with the test organism. It is rarely possible to seed a plate with a suspension of the test organism and drain off the excess fluid, as bacteria from the already developed colonies will be spread over the plate. On the other hand, to spread the suspension between the colonies by means of a loop or spreader without touching them is always tedious and frequently impracticable.

A satisfactory solution of the problem is to allow the potential antagonists to develop for a time, and then to spray a suspension of the test organism on to the plate. A device which we have used for this purpose is shown in Fig. 1. The conical flask *A* has a hole cut in the bottom slightly smaller than the Petri dish. The sprayer itself, the construction of which will be understood from the figure, is wrapped with cotton or cellulose tape and fitted snugly into the neck of the flask. Air is blown by means of a bulb through the cotton-wool filter *B* in tube *C*, the lower part of which is drawn out to a jet. Tube *D*, the top of which is sealed to *C*, is drawn out to a fairly fine point so that surface tension will prevent the few drops of bacterial suspension which are placed in *D* from running out by gravity; it must be accurately alined with *C* if a nebulous spray free from coarse droplets is to be obtained.

To use the sprayer, a few drops of bacterial suspension are placed in tube *D*, the flask *A* is placed on the Petri dish from which the cover has been removed, and the bulb is manipulated once or a few times. A beautifully even growth of bacteria is obtained if as little as one drop of suspension is sprayed in this way.

With highly pathogenic bacteria it is advisable to wrap the joint between flask and Petri dish with cotton-wool; also, sufficient time should be allowed for the fine droplets to settle before removing the flask from the dish.

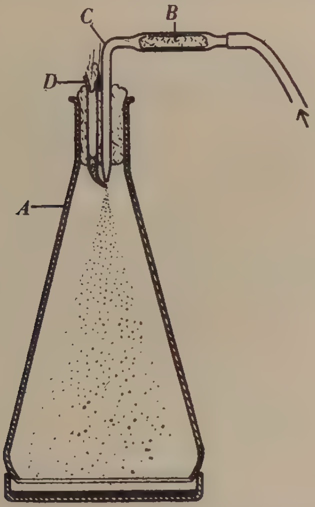

Fig. 1. Median vertical section through the spraying apparatus.

Plates seeded in this way require little or no drying. The technique has also been found convenient in the first stage in the search for antagonists, namely, for distributing the suspension of soil or other material under test. It is also suitable for surface seeding the agar for cylinder- or cup-plate assay.

(*Received* 3 *March* 1947)

The Production of stable Potent Preparations of Penicillinase

By E. S. DUTHIE

The Lister Institute, Elstree, Herts

SUMMARY: Optimal yields of penicillinase were obtained by the continuous addition of penicillin to cultures of certain strains of *Bacillus subtilis* during their logarithmic growth phase.

Lower yields of enzyme were obtained if the penicillin was added when growth had ceased or to partly grown cultures kept at rest and subsequently shaken.

Partial lysis of the bacteria was accompanied by an increased yield of enzyme, but complete lysis appeared to result in partial destruction.

Various authors, beginning with Abraham & Chain (1940), have shown that the ability of certain bacteria to destroy penicillin is due to the production of an enzyme penicillinase. Gram-positive bacteria of the *subtilis* group (cf. *Bacillus subtilis* or *B. licheniformis*, Duthie, 1944; Lawrence, 1944, 1945*a*, *b*; Ungar, 1944; *Bacillus cereus*, Benedict, Schmidt & Coghill, 1945; LePage, Morgan & Campbell, 1946) produce the enzyme in a stable extracellular form in considerable amounts, and culture filtrates of these organisms are therefore very suitable for removing penicillin in the sterility testing of penicillin batches and in recovering pathogenic bacteria from body fluids containing penicillin. In all cases it is important that the enzyme solution used should be of high potency, so that the penicillin present may be rapidly destroyed with as little attendant destruction as possible of any micro-organisms present.

In 1944 I showed that the addition of penicillin to cultures of *B. subtilis*, N.C.T.C. 6346, resulted in a 20–30-fold increase in the enzyme content of the culture fluid. Benedict, *et al.* (1945) obtained very high yields using strains of *B. cereus* grown in cultures of penicillin-producing strains of *Penicillium notatum* and *P. chrysogenum*. It was necessary to obtain a balance between the growth of bacteria and fungus, and the high yield was undoubtedly due to an enzyme adaptation as the result of penicillin production by the latter. Recently LePage *et al.* (1946) found that very high yields of penicillinase may be obtained by the gradual addition of penicillin in three stages to a previously aerated culture of the *B. cereus*, strain N.R.R.L. 569, used by Benedict *et al.* (1945). Growth was maintained for four days. The authors obtained partial purification of the enzyme by adsorbtion on to Hyflo Super-Cel (Johns Manville Ltd.), followed by elution with dilute ammonia and precipitation between 50 and 100% saturated ammonium sulphate.

More recently in two papers Housewright & Henry (1947*a*, *b*) have described their method of producing penicillinase from this same organism, N.R.R.L. B569. The yields of enzyme obtained were not very high, although they added large amounts of penicillin both initially and after 24 hr. growth.

My experiments over the past two years with two strains of *B. subtilis* have shown that penicillinase of high potency could be obtained consistently by the continuous addition of penicillin to shaken cultures, and large numbers of batches suitable for routine use have been made in this way. Although the enzyme yield is comparable with that obtained by Benedict *et al.* (1945) it is not as high as that claimed by LePage *et al.* (1946). On the other hand, the method is in many respects simpler than that used by either of these authors, since one single penicillin addition over a period varying from 2 to 24 hr. is sufficient and the results are very consistent. In addition, by varying the conditions it has been possible to make certain observations on the nature of the processes involved.

METHODS

Organisms used. Almost all the experiments were carried out with two strains of a *subtilis*-like organism, *B. licheniformis*, one the N.C.T.C. strain 6346 previously used, and another made available through the kindness of Mr R. F. Hunwicke and now entered in the National Collection of Type Cultures as strain no. 7198. This latter strain produced approximately twice as much penicillinase as did no. 6346 under all the conditions tested, and was equally constant in its behaviour. A comparison of the penicillinase-producing properties of five strains of *B. subtilis* and of seven strains of *B. licheniformis* obtained from the National Collection of Type Cultures showed that penicillinase production was not determined by the species. The highest yield of these twelve strains was given by *B. subtilis* N.C.T.C. 85, two strains of *B. subtilis* and five of *B. licheniformis* gave moderate yields, while two of each species gave little or none under the same conditions.

In addition, a few experiments were made with a strain of *B. cereus*, N.R.R.L. B 569, obtained from Dr R. G. Benedict, and used in his experiments (Benedict *et al.* 1945).

Culture methods. Since the majority of cultures were shaken at some stage in their growth it was found convenient to use either Ehrlenmeyer flasks containing about one-fifth of their volume of medium, or penicillin flasks with side spouts containing 200 ml. of the culture fluid. Continuous movement of the medium was secured in either case by placing the vessels on a horizontal table made to revolve eccentrically about 60 times/min. by means of a motor.

Since Lemco broth (% (w/v): peptone 1; Lemco meat extract 1; NaCl, 0·5) gave yields of enzyme which were consistently as high as those obtained with other media tested, this was used in all the experiments. The flasks were usually seeded and grown at rest for several days before experiments were made. Under these conditions shaking greatly increased the turbidity, bringing about a 5- to 10-fold increase in the number of bacteria present.

Penicillin was added continuously to penicillin flasks by means of a Eudrip no. 2 apparatus similar in principle to the no. 3 type described by McAdam, Duguid & Challinor (1944). In these experiments the penicillin flasks were fitted with rubber bungs containing two pieces of glass tubing, one plugged with wool and one covered with a rubber cap, and the penicillin was allowed to trickle in from the Eudrip apparatus through a hypodermic needle pushed through the rubber cap. The flasks and rubber bungs must be sterilized separately to prevent damage to the flasks.

Before assays of enzyme content were made, culture samples were freed of organisms by centrifuging, diluted 1/20 in neutral buffered gum arabic pH 7·0, and filtered in 8 ml. amounts through a 4 cm. Gradocol membrane of pore size about 0·64 μ. A number of samples could be filtered in succession through the same membrane with a consequent saving in time and expense. Large batches were successfully cleared by filtration through paper after the addition of Super-Cel and were then easily sterilized by filtration.

Estimation of penicillinase content. Two methods were used:

(1) Penicillinase to a final concentration of 1/20,000 was added to nutrient broth heavily seeded with the Oxford strain of *Staphylococcus aureus* and containing varying concentrations of penicillin such as 5, 10, 20, 50, 100 and 200 units/ml. The intermediate penicillinase dilutions were made in sterile nutrient broth and were incubated as a check on the sterility of the original enzyme solution. The mixtures of penicillin, enzyme and staphylococci were incubated for 24 hr. at 37°, and the activity of the enzyme was recorded as the number of units of penicillin destroyed by 1 ml. of 1/20,000 penicillinase; i.e. 0·0005 ml. enzyme as shown by the growth of the seeded staphylococci. This method was sometimes invalidated by the presence of chance contaminants and was therefore not as reliable as the second method.

(2) Falling dilutions of filtered penicillinase were added to 100 units of penicillin in M/150 phosphate buffer in 1 % gum arabic at pH 7·4. The mixtures were incubated for 1 hr. at 37°, after which samples were placed in cylinders on staphylococcal seeded plates as in the penicillin assay method described by Heatley (1944) and placed in the incubator. All dilutions were made in sterile solutions, although this is probably not necessary, and results were expressed as the smallest volume of the original solution which brought about complete destruction of the penicillin as shown by the absence of an inhibition zone. The method is similar to that described by the present author in 1944 and used subsequently by various workers, although the penicillin concentration and time of incubation is different.

RESULTS

Influence of adding penicillin continuously. In previous studies (Duthie, 1944) it was demonstrated that the addition of penicillin to a fully grown culture of *B. subtilis* 6346 grown at rest increased the yield of penicillinase some 20- to 30-fold. The only effect of continuous shaking following or during the addition of penicillin was to accelerate the rate of formation of penicillinase without appreciably increasing the final yield. In these early experiments the penicillin was added in single amounts after 12, 24 or 48 hr. growth. Since it was obvious that under these conditions penicillinase was present in sufficient amounts to destroy the added penicillin very rapidly and increasing the amount of the penicillin added in this way did not affect the final yield, the effect of adding penicillin continuously over 12–24 hr. was now investigated with the results shown in Table 1.

Table 1. *Effect of adding* 10,000–15,000 *units penicillin in* 100 *ml. saline in various ways on the production of penicillinase from* Bacillus licheniformis, *N.C.T.C. strains* 6346 *and* 7198

All cultures were grown in 200 ml. Lemco broth 3–5 days at rest before the addition of penicillin. Temperature 22–26°.

Penicillin added	Condition during addition	Units of penicillin inactivated by 0·00005 ml. enzyme solution in 24 hr. at 37°. Method 1	Vol. of enzyme solution required to inactivate 100 units penicillin in 1 hr. at 37°. Method 2 (ml.)
Continuously	Shaken	100–150	0·0002–0·0015
Continuously	Rest	6	—
In one lot at beginning of shaking	Shaken	20	—
6 lots over 36 hr.	Shaken	20	0·004
6 lots over 36 hr.	Rest	5	—
No penicillin added	Shaken	<0·5	—

It will be seen that when penicillin is added continuously and the culture is agitated during its addition the final yield of enzyme is much greater than that obtained in any other way, being some ten to twenty times as powerful as when the penicillin is added in one single amount at the beginning of shaking or in six equal amounts over a 36 hr. period. Cultures kept at rest yielded even less.

Table 2. *Penicillin in* 100 *ml. saline added continuously to* 200 *ml. of a shaken broth culture of* Bacillus licheniformis, *N.C.T.C.* 6346 *at* 26°

Culture grown at rest. Shaken during penicillin addition and allowed to lyse later.

Units penicillin added	Units penicillin inactivated completely by 0·0005 ml penicillinase in 24 hr. at 37°. Method 1
100,000	6
50,000	25
20,000	50–100
10,000	50–100
5,000	12
None	2

Amount of penicillin added and time of addition. In all the experiments in Table 1, 10,000–15,000 units of penicillin in 100 ml. of saline were added and (Table 2) this was optimal for the amount of culture used (200 ml.). This final value of 50 units penicillin/ml. of culture is curiously enough similar to that adopted by LePage *et al.* (1946), but is one-eighth of that added by Housewright & Henry (1947*a*). A few experiments showed that the period over which penicillin is added continuously should lie between 2 and 24 hr. and that there was little advantage in prolonging the time over the 24 hr. period.

Treatment of cultures before, during and after the addition of penicillin. The highest and most constant yields were obtained when penicillin was added continuously to a culture first grown at rest and then shaken to ensure further growth during the addition of penicillin (Table 3). The yields were sometimes as high, but were less consistently so when penicillin was first added some 12 hr. after shaking had begun, by which time the culture had reached maximum growth. Much lower yields were obtained if the penicillin was added to an unshaken (partly grown) culture at rest and the culture shaken subsequently. After shaking and adding the penicillin it was found advantageous to leave the cultures at rest for several days at 26° (Table 4) or for 6–12 hr. at 37°. During this time lysis occurred most completely and rapidly in those flasks to which penicillin had been added. Cultures of no. 6346 became almost clear, and those of no. 7198 showed partial clearing. It was noted that complete clearing in the case of 7198 resulted in a lower yield of penicillinase than that obtained if only partial lysis occurred, suggesting that while lysis may result in the liberation of the enzyme it may also bring about its partial destruction.

Table 3. *Effect of adding penicillin to different growth stages of culture on enzyme yield*

Strain no. 7198. Temperature 37° Condition of growth			Filtrate concentration giving complete destruction of 100 units/ml. in 1 hr. at 37°. Method 2
Before penicillin addition	During penicillin addition	After penicillin addition	
(1) Rest 18 hr.	Rest 18 hr.	Shaken 12 hr.	<1/500
(2) Rest 18 hr.	Shake 12 hr.	Rest 6 hr.	1/2000
(3) Shake 18 hr.	Rest 12 hr.	Measured at once	1/1000
(4) Shake 18 hr.	Shake 12 hr.	Rest 6 hr.	1/1000

Table 4. *Effect of lysis on penicillinase yield strain, N.C.T.C.* 6346 *at* 26°

Culture grown at rest; shaken during penicillin addition and allowed to lyse later.

Units penicillin added	Units penicillin inactivated by 0·0005 ml. penicillinase in 18 hr. at 37°. Method 1		
	Measured immediately	After 24 hr. rest or shaken	After 7 days at rest
20,000	12·5	50	100
10,000	12·5	50	100
5,000	12·5	12·5	100

Temperature. Equal enzyme yields were obtained at 26 and at 37°, but the former was preferred, since it gave more consistent results. The periods needed to obtain optimal results were in general much shorter at 37°, and Table 5 shows those periods which gave optimal results for the temperatures indicated.

The amount of inoculum used did not matter within wide limits provided that before the addition of penicillin growth was sufficiently vigorous to ensure that it was not suppressed by the addition of the antibiotic.

Media. Equally good results were obtained on infusion digest and on a meat-extract broth such as Lemco. An acid-hydrolyzed casein medium similar to that used by Rogers (1945) gave about half the yield obtained on the other media and the enzyme was less stable when diluted. The addition of glucose to the Lemco broth medium used was without effect.

Deep fermentation. Heavy suspensions of both *subtilis* strains were obtained by the continuous passage of air through a 12 l. bottle containing 4 l. of Lemco medium. The suspensions failed to lyse although penicillin was added continuously during the growth phase, and neither the culture fluid nor the crushed organisms gave a high yield of penicillinase.

Table 5. *Conditions for optimal production of penicillinase*

10,000 units penicillin added continuously to 200 ml. medium.

Temperature ° C.	Period of growth at rest	Duration of penicillin addition	Period of incubation after penicillin
37	12–24 hr.	6–24 hr.	6–12 hr.
26	2–5 days	12–36 hr.	2–7 days

Organisms. As previously noted, *B. licheniformis*, strain 7198, gave approximately twice the penicillinase yield given by 6346 under identical conditions, and this appeared to be correlated with the fact that it lysed less completely. The strain *B. cereus*, N.R.R.L. B569, used by Benedict *et al.* (1945) gave approximately one-twentieth of the maximum given by no. 7198 on the Lemco broth medium. The continuous addition of penicillin varying from 250 to 10,000 units to 200 ml. of medium to a penicillinase-producing strain of *Staphylococcus* did not markedly increase the penicillinase content of the culture fluid.

Comparison of methods of measurement. The methods of assaying penicillinase used by various authors differ so widely through variation of substrate concentration, time and temperature, that it is quite impossible to compare the potency of the different preparations recorded without carrying out a number of experiments. Probably the simplest, if not the most accurate, is to incubate various dilutions of penicillinase with a constant amount of penicillin, and at the end of a given time to assay the solutions for penicillin activity on staphylococcal seeded Petri dishes. The titre may then be taken as the lowest concentration of enzyme giving complete destruction or 50 % destruction of the penicillin. The latter is less easily determined, especially if a biological assay method be the only one available. In the present experiments 100 units/ml. was the level of penicillin adopted, but if this concentration be varied, as in Table 6, it will be seen that a much higher substrate level than 100 units/ml. is required in order to reach full activity of the enzyme using either the 50 or 100 % level as an index of enzyme activity. Thus at a penicillin concentration of 100 units/ml., about half as much penicillin is destroyed by every ml. of enzyme solution as at 1000 units/ml.

Benedict *et al.* (1945) found that 0·002 ml. of their culture filtrates completely destroyed 100 units of penicillin in 1 ml. in 4 hr. at 30°, and the batch used in the measurements in Table 6 had approximately the same activity under these conditions. When dialyzed against distilled water 0·0001 mg. of this preparation destroyed 50 % of 100 units of penicillin in 1·0 ml. of phosphate buffer gum arabic pH 7·0 in 3 hr. at 30°, which compares favourably with the preparation described by Benedict *et al.* (1945). On the other hand, LePage *et al.* (1946)

found that some of their culture filtrates at 1/1,000,000 gave a 50 % reduction of penicillin solutions in final concentration of 1 unit/ml. in 1 hr. at 37° and were therefore some 25 times as active as was the preparation used in Table 6.

Table 6. *The influence of the concentration of penicillin on the activity of penicillinase*

Penicillin concentration (units/ml.)	Enzyme concentration giving complete destruction in 1 hr. at 37°. Method 2	Approx. enzyme concentration giving 50 % destruction in 1 hr. at 37°
1	1/20,000	1/40,000
10	1/5,000	1/20,000
100	1/2,000	1/8,000
1000	1/500	1/2,000
5000	1/100	—

Recently, Housewright & Henry (1947*a*, *b*) have shown that penicillinase activity can be measured by following the rate of carbon dioxide evolution from bicarbonate solutions in Warburg manometer vessels to which mixtures of penicillin and penicillinase have been added. In addition, these authors have standardized their enzyme yields biologically by two methods: (*a*) the destruction of 10 units of penicillin per ml. in broth in 48 hr. at 37° as shown by the development of anthrax spores (maximum titre obtained = 1/8192); and (*b*) the destruction of 57·5 % of 50 units penicillin in 11 ml. at pH 7·0 in 1 hr. at 37°, the solutions being diluted and plated (method of McQuarrie & Liebmann (1944) (titre of maximum yield obtained = 1/1400)). Under these conditions the preparation measured in Table 5, which had about half the activity of the best preparations obtained, gave values of 1/150,000 and 1/40,000 respectively, indicating that it was at least twenty times as potent as those obtained by Housewright & Henry. The destruction of penicillinase by Fe^{+++} ions noted by these authors (between 5 and 50 μmg./ml. levels) has been confirmed. Fe^{++} ions have a similar, but less marked action.

DISCUSSION

There is little doubt that organisms of the mesentericus-subtilis group are the most potent source of stable cell-free penicillinase, and it is therefore of some importance to learn the conditions under which a highly active penicillinase may be obtained. The formation of the enzyme by these organisms is largely adaptive and would appear to depend on the presence of active substrate in the culture for a period of time varying from 2 to 24 hr. during, or at the end of, the logarithmic growth phase. Since penicillin is rapidly destroyed by pure enzyme in the culture medium, substrate must be added continuously in order to produce optimal yields. Benedict *et al.* (1945) obtained this by growing the bacteria in contact with penicillin-producing fungi and LePage *et al.* (1946) state that penicillin was added gradually to their cultures on three different occasions.

The value of shaking the cultures before or during the addition is clear, since the greatly increased cell population placed in contact with substrate gives a higher penicillinase yield than in those left at rest. It is interesting, in this connexion, that the addition of penicillin to resting cells subsequently shaken does not result in a great increase in the yield of enzyme; there is, therefore, no permanent adaptation transmissible to the daughter cells.

Unfortunately, the relation of enzyme production to lysis is by no means so clear. LePage *et al.* (1946) noted a fall in the enzyme titre of lysed cultures followed by a rise 24 hr. later, and add that if lysis is observed during the addition of penicillin no more penicillin should be added for a further 24 hr. In the present studies, while it was obvious that some degree of lysis enhanced the penicillin content of the medium, complete lysis was accompanied by a fall and partial lysis gave the highest yields.

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The Production by Certain Species of *Clostridium* of Enzymes Disintegrating Hide Powder

By D. G. EVANS

National Institute for Medical Research, Hampstead, London

SUMMARY: The production by certain species of *Clostridium* of enzymes disintegrating hide powder was investigated by measuring the lytic action of broth cultures and toxic filtrates on finely divided hide powder suspended in an agar gel.

Cl. histolyticum was the most active producer of enzyme, *Cl. welchii* A was less active and *Cl. sporogenes* and *Cl. bifermentans* only moderately active. *Cl. tetani*, *Cl. oedematiens* and *Cl. septicum* produced no such enzyme. The lytic enzyme of *Cl. histolyticum* is not the lethal toxin.

Among strains of *Cl. welchii* type A, enzyme production, α-toxin production and ability to cause fatal infection in guinea-pigs are associated.

There was some evidence that the different enzymes affecting hide powder are antigenically related, but no definite conclusion is possible, since the antisera employed may have contained antibodies to the lytic enzymes of a number of different organisms.

Maschmann (1938) claimed that *Cl. welchii* elaborated an enzyme which splits collagen. This he named collagenase but recorded no experiments demonstrating its specific action. Later Jennison (1945) recorded the disintegration of collagen fibres by enzymes produced in actively growing cultures of aerobic and anaerobic organisms. Macfarlane & MacLennan (1945), confining their study to *Cl. welchii* Type A, found that culture filtrates were able to break down muscle by their action on the collagen of the reticulin scaffolding. They regarded the active substance as a collagenase and suggested that it was responsible for the muscle destruction observed in cases of gas gangrene caused by *Cl. welchii* A and thus played an important part in the pathogenesis of the disease. The collagenase of *Cl. welchii* A was shown by Oakley, Warrack & van Heyningen (1946) to be immunologically distinct from the other known antigens present in culture filtrates, and *Cl. welchii* A antisera contained a distinct anticollagenase. Evans (1945*b*, 1947) showed that the anticollagenase alone had no protective action in experimental *Cl. welchii* A infections, and concluded that although collagenase may be responsible for the muscle destruction observed in *Cl. welchii* A infections, there was no evidence to suggest that it had any significant role in determining whether fatal infection would follow the injection of a given inoculum of *Cl. welchii* A.

The strains of *Cl. welchii* A used in these experimental infections all produced collagenase *in vitro*, though in variable amounts. Oakley *et al.* (1946), in estimating the anticollagenase potency of *Cl. welchii* antisera, obtained identical results using as substrate either collagen prepared from horse tendon or commercial hide powder. Hide powder, although not a pure substrate, is a rich and convenient source of collagen and was employed in the tests described below. The disintegration of hide powder, however, is not a specific indication of collagenase action but provides reasonable presumptive evidence that a collagenase is present. A study has been made of the *in vitro* production by a number of strains of *Cl. welchii* A and by other species of *Clostridium*, of enzymes with ability to disintegrate hide powder.

METHODS

The production of lytic enzymes by *Cl. welchii* A was first demonstrated by incorporating the hide powder in the culture medium. Hide powder was passed through a sieve, 60 meshes to the inch, dried over P_2O_5 *in vacuo* for 2 days and sterilized by dry heat at 100° for 1 hr. To 100 ml. of saline 4 g. of the hide-powder preparation were added and a smooth suspension obtained by vigorous agitation. This was added to melted Fildes's agar medium, 12 % by volume, and pour-plates made in which the hide-powder particles were distributed densely and uniformly. Sterilization of the hide powder was necessary to eliminate contaminants, but it is improbable that the dry heating caused significant degradation of the collagen, for similar results were obtained with unsterilized hide powder.

After 24 hr. anaerobic growth on this medium strains of *Cl. welchii* A gave colonies surrounded by a zone in which complete dissolution of the hide powder had occurred and which increased in size after a further 24 hr. incubation. With different strains the size of the zone of clearing varied from a faint narrow zone to one three times the diameter of the colony.

The method was, however, unsuitable for comparing the lytic enzyme production of different strains of *Cl. welchii* A, or for detecting lysis by species of *Clostridium* that grow in widely spreading colonies. To obviate these difficulties, the amount of enzyme produced in broth cultures was titrated in cups cut in non-nutrient hide powder-agar plates. These plates were made by adding 2 ml. of the saline hide-powder suspension to 15 ml. of melted 4 % agar in saline and pouring the mixture into a Petri dish. Cups of 7 mm. diameter were cut with a cork-borer, and the amount of enzyme in a culture was then titrated by pipetting into each cup approximately 0·1 ml. of falling dilutions of an 18 hr. culture in rabbit-liver broth and incubating aerobically at 37°. After 24 hr., actively lytic strains gave around the cup a distinct concentric zone completely free from hide-powder particles; the zone increased in size after a further 24 hr. incubation, when the final readings were made. There was no disadvantage in filling the cups with whole-broth culture, since there was no possibility of the growth of anaerobes occurring on this medium. Moreover, tests made with the clear supernatant fluid from centrifuged cultures gave results identical with those using whole culture.

RESULTS

Lysis of hide powder by Clostridium welchii *A*

All the thirty strains of *Cl. welchii* A which were used in a previous investigation (Evans, 1945*a*), and which had been preserved in the dry state in the meantime, were found to produce enzymes disintegrating hide powder. The zone of clearing was 25 mm. in diameter with the most active strains and less than 10 mm. in diameter with the least active. The strains were classified into three groups according to the diameter of the zone and arranged in order of α-toxin production (Table 1). Strongly lytic cultures were active when diluted 1:100, but those which gave small zones were inactive when diluted 1:4 and in some cases 1:2. When enzyme production by the thirty strains of *Cl. welchii* A is compared (Table 1) with α-toxin production and the ability to cause fatal infection in guinea-pigs (Evans, 1945*a*), it is evident that there is a general association between these three properties.

Table 1. *Comparison of hide-powder enzyme production with other properties of thirty strains of* Clostridium welchii *A*

Enzyme production: + = <10 mm., ++ = 10–15 mm., and +++ = >15 mm. zone of hide-powder clearing.

α-toxin production: figures = number of units of α-antitoxin required to neutralize the α-toxin in 1 ml. of culture.

Virulence for guinea-pigs: + = virulent; − = avirulent.

	In vitro production of		
Strain	Hide-powder enzyme	α-toxin	Virulence for guinea-pigs
S107	+++	1·60	+
SR12	+++	0·95	+
A119	+++	0·85	+
A117	+++	0·60	+
A118	+++	0·60	+
SR9	+++	0·35	+
Rosher	+++	0·25	+
G5*g*	+++	0·25	+
3893	+++	0·20	+
S1*a*	++	0·20	+
BB	+++	0·20	+
BS1	+++	0·20	+
3895	++	0·15	−
26	+++	0·15	+
A102	+	0·15	−
274	++	0·10	+
Mills	++	0·10	+
7731	++	0·10	+
A19	++	0·10	±
PL	+	0·05	+
5053	++	0·05	+
529	++	0·03	−
P5706	+	0·03	−
Corcoran	++	0·03	−
D5	++	0·03	−
4226	+	0·02	−
A78*b*	+	0·02	−
A38*b*	+	0·02	−
D3*a*	+	0·02	−
A13*b*	+	0·02	−

The zones of hide powder clearing were evidently produced by a true collagenase, since the activity of the cultures was neutralized by three of Dr Oakley's *Cl. welchii* A antisera in proportion to their anticollagenase content. (1) Serum R5434 contained 50 units (Oakley, *et al.* 1946) of anticollagenase and 0·2 unit of α-antitoxin per ml., (2) serum R6423 contained 75 units of α-antitoxin per ml. but no detectable anticollagenase, and (3) serum Ex1055 contained 2500 units of anticollagenase and 500 units of α-antitoxin per ml. With each of the thirty strains of *Cl. welchii* A, mixtures of equal parts of an 18 hr. culture and each serum were held for 1 hr. at room temperature and then pipetted into the hide powder-agar-cup plates, which were incubated at 37° for 48 hr. Serum R6423 had no effect on the zones of clearing produced by each of the thirty strains; the zones were similar in size to those produced by mixtures of culture and normal horse serum. On the other hand, serum R5434 completely inhibited the clearing produced by twenty-one of the strains; the remaining nine strains, all of which produced large zones, were partly neutralized, 20 mm. zones being reduced to less than 12 mm. in diameter. The more potent anticollagenase serum Ex1055 completely neutralized the clearing by all thirty strains.

Lysis of hide powder by other species of Clostridium

The following strains were tested for enzyme production: *Cl. sporogenes*: M1*f*, M41*a*, M3*g*, M5*f*; *Cl. oedematiens*: H1, Jolly, M131*o*, M4*t*; *Cl. bifermentans*: M74, M16, M15*h*, M58*e*, M1*e*; *Cl. septicum*: VS189, VS54, M16*m*, M40*f*; *Cl. tetani*: T67, T279; *Cl. histolyticum*: CN1693, CN950, CN919, CN920, CN949; and a non-pathogenic *Clostridium* with morphological and biochemical properties almost identical with those of *Cl. histolyticum* but which did not produce *histolyticum* toxin: strains M37*e*, M55, M17*l*.

The M strains were isolated from war wounds by Mrs E. M. Miles, and the *Cl. histolyticum* strains were supplied by Miss Helen E. Ross of the Wellcome Physiological Research Laboratories. The strains had been preserved either in alkaline-egg medium or in the dry state, and were subcultured a number of times to bring them into an actively growing condition. For the titration of enzyme each strain was grown in rabbit-liver broth for 18–20 hr.

Cultures of all the *Cl. histolyticum* strains produced rapid and complete dissolution of hide powder; even after 3 hr. a distinct rim of clearing was visible around the cups and at 24 hr. the diameter of the zone was 20 mm., increasing to an average of 30 mm. after a further 24 hr. incubation. The titres of these cultures were as high as 1:250. Each of the five strains of *Cl. histolyticum* produced, in guinea-pigs, an infection showing extensive muscle destruction which, with the infecting doses employed, was not always accompanied by the death of the animal.

Cultures of *Cl. bifermentans*, *Cl. sporogenes* and the three *Cl. histolyticum*-like strains also contained enzymes disintegrating hide powder. Their behaviour, however, differed from that of *Cl. welchii* A and *Cl. histolyticum* cultures. Although the zones of clearing at 24 hr. were distinct and 15–20 mm. in diameter, the clearing was only partial. Large numbers of unchanged hide-powder particles were visible within the zones, while other particles appeared to be only slightly affected. After a further 24 hr. incubation, the zones became completely clear, although they were not well defined. The concentration of enzyme in these cultures was not high, for dilutions of 1:32 had little or no activity. The low concentration may have been responsible for the slow dissolution of the hide powder, for a concentrated filtrate of *Cl. bifermentans* culture produced in 24 hr. a zone completely free from hide-powder particles.

No enzyme attacking hide powder was demonstrable in the cultures of *Cl. oedematiens*, *Cl. septicum* and *Cl. tetani*. Two strains of each species proved to be highly pathogenic for guinea-pigs, so that with these organisms at least, virulence and enzyme production are not associated.

Some of Jennison's (1945) results have been confirmed in this investigation. He found that cultures of *Cl. histolyticum*, *Cl. sporogenes* and *Cl. bifermentans* disintegrated collagen fibres and that *Cl. histolyticum* was the most active. He reported, however, that *Cl. welchii* did not affect the collagen substrate, a result which may well have been due to poor collagenase production by the strains of *Cl. welchii* he used.

Lysis of hide powder by toxic filtrates

A number of dry preparations of ammonium sulphate precipitates from toxic filtrates of various organisms of the gas gangrene group, were tested by the cup-plate method for enzyme activity. Forty mg. of each toxin were dissolved in 1 ml. of saline, giving solutions containing many mouse lethal doses/ml. The toxic precipitates from *Cl. tetani*, *Cl. oedematiens* and *Cl. septicum* contained no enzyme which disintegrated hide powder, whereas those of *Cl. histolyticum* and *Cl. welchii* A produced clear wide zones in less than 24 hr. By titration it was found that the smallest concentration of toxin showing enzyme activity (M.E.D.) was 0·5 mg./ml. with the most potent *Cl. welchii* A preparation and 0·004 mg./ml. with the most potent *Cl. histolyticum* toxin.

Five different dry ammonium sulphate precipitates, prepared from *Cl. histolyticum* (I–V) in different institutes throughout the world, were titrated in parallel for enzyme activity and lethal power on intravenous injection in mice (Table 2). It is evident from the inconsistent ratios of enzyme activity to toxicity that the factors responsible for these two effects are not the same.

Table 2. *Hide-powder enzyme activity and toxicity of* Clostridium histolyticum *toxins*

Histolyticum toxin sample	Minimal enzyme concentration (mg./ml.)	Minimal lethal dose for mice (mg.)	Ratio M.E.D.:M.L.D.
I	1/256	1/32	8:1
II	1/16	1/32	1:2
III	1/128	1/8	16:1
IV	1/64	1/16	4:1
V	1/128	1/8	16:1

Neutralization of enzyme by antisera

The cup-plate method was also used in an attempt to investigate the antigenic relationships of the enzymes attacking hide powder produced by the different organisms. Three antisera were employed: (1) *Cl. welchii* A horse antitoxin Ex1055, (2) the International Standard *Cl. histolyticum* antitoxin reconstituted so that 280 units of antitoxin were contained in 1 ml.; this antiserum was also from a horse, and (3) *Cl. bifermentans* rabbit antiserum 2718 (Miles & Miles, 1947). Each serum was titrated for neutralization property with a number of homologous and heterologous enzyme preparations. Twofold dilutions of antiserum were made, and to each dilution an equal volume of the enzyme preparation was added. In most tests the mixtures were so constituted that the concentration of enzyme in each cup was eight times the smallest concentration which gave a reaction (8 M.E.D.). It was, however, necessary in some cases, where neutralization was only slight, to diminish the concentration of enzyme in the cup in order to obtain an end-point in the titration.

Table 3. *Neutralization of hide-powder enzyme by various antisera*

The figures indicate the reciprocal of the serum dilution, and the figures in brackets the number of minimal enzyme doses (M.E.D.) used in the titration.

Enzyme preparation		Neutralization titres of antisera		
		welchii (Ex1055)	*histolyticum* (Int. St.)	*bifermentans* (2718)
Cl. welchii:	Toxin Welchpool	2048 (8)	32 (8)	<2 (2)
	Culture Rosher	2048 (8)	32 (8)	<2 (2)
Cl. histolyticum:	Toxin I	2 (4)	512 (8)	<2 (2)
	Toxin II	2 (4)	1024 (8)	<2 (2)
	Culture CN920	2 (4)	512 (8)	<2 (2)
	Culture CN1693	2 (4)	512 (8)	<2 (2)
Cl. bifermentans:	Culture M58*e*	64 (8)	32 (8)	128 (8)
	Culture M15*h*	32 (8)	16 (8)	64 (8)
Cl. sporogenes:	Culture M1*f*	32 (8)	8 (8)	2 (8)
	Culture M5*f*	32 (8)	8 (8)	4 (8)

Some evidence was obtained (Table 3) of an antigenic relationship between the enzymes of the different organisms. There were, however, inconsistencies in the results, such as the low titres of *welchii* antiserum with *histolyticum* antigens compared with the relatively higher titres of *histolyticum* antiserum with *welchii* antigens, and also the inability of the *bifermentans* antiserum to show cross-reactions with either *welchii* or *histolyticum* antigens, although both *welchii* and *histolyticum* antisera reacted with *bifermentans* enzyme. It was, of course, possible that the horse antisera contained antibodies to the lytic enzymes of a number of different organisms, naturally produced in the animals from which the sera were obtained. Nothing was known of the anti-enzymic properties of the sera of the animals before they were used for preparing the antisera, and there was evidence that the *histolyticum* serum came from a horse with some experience of *Cl. welchii* A antigens. More was known of the *bifermentans* antiserum since it was prepared in a rabbit which had received only *Cl. bifermentans* culture, and its greater specificity may have been a result of this, although, on the other hand, it may have been due to its low homologous titre. It is clear that if the antigenic relationship of the enzymes produced by the different organisms were to be investigated, it would be necessary to prepare antisera in selected animals whose sera before immunization were entirely free from antibodies to enzymes disintegrating hide powder.

DISCUSSION

From the results of this investigation it may be stated that the ability of various species of *Clostridium* to elaborate enzymes affecting hide powder is not related to the pathogenicity of the species, for both pathogenic and non-pathogenic organisms produce enzymes which disintegrate hide powder and, furthermore, a number of pathogenic species of *Clostridium* show no such enzyme activity. It is noteworthy, however, that of the pathogenic organisms examined, *Cl. welchii* A and *Cl. histolyticum*, both of which elaborate the lytic enzyme, produce lesions with extensive muscle destruction, whereas muscle disintegration does not occur in lesions caused by *Cl. tetani*, *Cl. oedematiens* and *Cl. septicum* which do not produce the enzyme.

There is clearly an association between enzyme production and virulence of strains of *Cl. welchii* A, but so far there is no evidence to suggest that the ability to produce enzymes disintegrating hide powder in any way determines virulence, which appears to depend mainly on the production of the lethal α-toxin (Evans, 1945*b*, 1947).

The part played by the antigens of *Cl. histolyticum* in infections produced by this organism has not been so fully investigated, but *Cl. histolyticum* produced in guinea-pigs a non-fatal infection with extensive muscle destruction. It is reasonable, therefore, to suppose that the enzyme disintegrating hide powder is associated with muscle destruction, and that when fatal infection occurs it is probably due to the lethal toxin of *Cl. histolyticum*.

I am greatly indebted to Dr C. L. Oakley for supplies of *Cl. welchii* A antisera and also to Miss Helen E. Ross and Mrs E. M. Miles for some of the strains.

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The Lecithinase of *Clostridium bifermentans* and its Relation to the α-toxin of *Clostridium welchii*

By ELLEN M. MILES and A. A. MILES

National Institute for Medical Research, Hampstead, London

SUMMARY: *Clostridium bifermentans* elaborates a lecithinase which induces turbidity and curding in crude lecithovitellin preparations, and hydrolyzes egg-yolk lecithin with the liberation of acid-soluble phosphate, both over a pH range of 5·0–6·3 with a maximum in the region of pH 6·0. It does not induce turbidity in human serum alone, but does so in the presence of agar gels, which activate the reaction over a pH range of 5·0–8·0. The activities are not dependent on the presence of Ca^{++} ions. The enzyme responsible appears to be a lecithinase C.

In the concentration available the lecithinase haemolysed rabbit and mouse, but not human, horse, sheep or guinea-pig red cells; and it was not lethal on intravenous injection into mice, but was slightly toxic in the skin of guinea-pigs.

The *Cl. bifermentans* lecithinase C is antigenically related to the lecithinase C (the α-toxin) of *Cl. welchii*, though differing from the α-toxin in its pH optima, haemolytic powers, independence of Ca^{++} ions and relative non-toxicity. It is neutralized by *Cl. welchii* α-antitoxin, and *Cl. bifermentans* antilecithinase behaves in a reciprocal manner, neutralizing the lecithinase, necrotizing and lethal action of *Cl. welchii* α-toxin. The antigenic relation of the two lecithinases appears to be remote.

Several species of *Clostridium* elaborate substances that react with normal human serum to produce turbidity or a curd of fatty material. This reaction was described by Nagler (1939) for *Cl. welchii* type A; in this case it is due to a lecithinase apparently identical with the α-toxin of that species (Macfarlane, Oakley & Anderson, 1941; Macfarlane & Knight, 1941; van Heyningen, 1941), and the lecithinase is specifically inhibited by α-antitoxin. The reaction may also be observed in plate cultures on agar containing human serum; a zone of turbidity develops round the growing colony. Hayward (1943) devised the plate reaction for the identification of *Cl. welchii* in mixed cultures, half the plate being impregnated with *Cl. welchii* antitoxin to distinguish *Cl. welchii* from other Nagler-reacting organisms. Inhibition by the antitoxin was not, however, fully specific, for the zones produced by *Cl. bifermentans*, *Cl. sordellii* and *Cl. centrosporogenes*, unlike those of other Nagler-reacting bacilli, were substantially neutralized by the antitoxin. Indeed, the inhibition by *Cl. welchii* antitoxins of a Nagler zone round colonies of readily sporing anaerobic bacilli proved to be a useful preliminary identification of *Cl. bifermentans* in mixed cultures from wounds.

Most strains of *Cl. bifermentans* are neither toxigenic nor pathogenic to laboratory animals; yet they appear to elaborate a lecithinase-like substance that is related antigenically to the main toxin of *Cl. welchii*. The substance invited investigation, not least in the hope of illuminating the relation of lecithinase activity to toxicity in the α-toxin of *Cl. welchii*.

Our results strongly suggest that the Nagler-reacting substance of *Cl. bifermentans* is in fact a lecithinase, and it will be convenient to call it so from the outset.

MATERIALS AND METHODS

Crude bifermentans *lecithinase* (*BL*). Strain M58*e* of *Cl. bifermentans*, isolated from a war wound, was selected for its strong Nagler reactivity on human serum plates. With egg-yolk extract as prepared by Crook (1942) as indicator, and M/15-borate buffer pH 7·2, no lecithinase was detectable in cultures of M58*e* in ox-heart extract broth, cooked meat medium, or Brewer's (1940) medium. A lecithinase was produced in Macfarlane & Knight's (1941) glucose peptone muscle-extract medium, and in rabbit liver broth (McLeod, J. W., personal communication). McLeod's broth is a meat-extract broth to which about 10% of parboiled minced rabbit liver is added before the final autoclaving. It was chosen for lecithinase production because anaerobiosis is achieved in it without excluding air from the container. The lecithinase produced was optimally active between pH 2·3 and 4·5, and was resistant to 100° for 10 min. This proved to be a mixture of the *bifermentans* lecithinase with another lecithinase-like substance, and uninoculated McLeod's medium also proved to contain a heat-stable substance of the same kind capable of curding yolk extract. It was apparently released from the fragments of rabbit liver during sterilization by autoclaving, for a similar substance was obtained from parboiled mince of rabbit liver. Macfarlane & Knight's medium, being free from lecithinase in the uninoculated state, was therefore adopted for lecithinase production. For bulk preparation, the broth at 37° was seeded with one-twentieth of its volume of a vigorously growing 24 hr. cooked-meat medium culture of M58*e*. Lecithinase production was maximal between 8 and 18 hr. at 37°. The culture was usually harvested at 18 hr., partly cleared by centrifugation and filtered through a Berkefeld candle. The filtrate, which contained from 16 to 32 arbitrary lecithinase units/ml. (see p. 387), was concentrated fourfold by dialysis against glycerol in cellophan sacs, and the glycerol partly removed from the concentrate by subsequent dialysis against saline. The residual glycerol, left as a preservative, had no effect on the lecithinase activity, except slightly to enhance the rate of formation of the curd in yolk extract.

The most concentrated preparation, containing 1024 arbitrary units/ml., was made by Dr E. S. Duthie by pressure dialysis of Berkefeld culture filtrates in Visking sausage casing, followed by dialysis against distilled water for 24 hr. at 0–4° and preserved with toluene. These preparations were stable for several months at 2°. *Bifermentans* lecithinase is symbolized in the text as BL.

Crude welchii *lecithinase* (*WL*). In all, three different preparations of crude culture filtrates from *Cl. welchii* type A were used, each from a different strain of the organism. They were concentrated by dialysis against pure glycerol at 3°. *Welchii* lecithinase is symbolized in the text as WL.

Titrations of bifermentans *and* welchii *lecithinases* (*BL and WL*). Lecithinase activity was titrated in 8 × 1 cm. tubes at 37°; they contained four 0·25 ml. volumes of reagents: BL or WL preparation; 0·85% saline or antiserum diluted in saline; M/15-acetate buffer pH 5·1; yolk extract (Crook, 1942). With weak solutions of BL, a faint turbidity developed in 3–4 hr., and curding was complete at 18 hr. The end-point was not sharp; over a four- to eightfold range of diminishing concentration, the reaction at 18 hr. declined from a thick curd floating on a clear subnatant fluid, through lesser curds on a turbid fluid, general flocculation, to faint turbidity. This gradual dose response was, however, relatively constant.

With WL, maximum titres were obtained at 6 hr.; the titre was not significantly increased at 18 hr. The dose response was sharper, the 0–100% reactions being covered in a twofold range of WL concentrations.

The unit of lecithinase (the BL and the WL unit) in both cases was arbitrarily defined as the smallest amount which, in the titration mixtures described above, would produce complete separation of the curd in 18 hr. at 37°; e.g. a lecithinase preparation of which 0·25 ml. produced a full curd at 1/16 contains 64 units/ml. Our WL unit corresponded approximately to 0·1 mouse M.L.D. of toxin and to approximately one-twentieth of Macfarlane & Knight's (1941) arbitrary lecithinase unit (see Table 3). In some simultaneous titrations, it was more convenient to use as end-point, not complete curding, but the smallest visible trace of turbidity in yolk extract. Owing to the different dose responses to BL and WL, these end-points, though in each case constantly related to the unit, are not directly comparable in terms of units.

PROPERTIES OF THE *CLOSTRIDIUM BIFERMENTANS* LECITHINASE

Lecithinase activity. The concentrated broth filtrates from *Cl. bifermentans* acted upon yolk extract, human serum, and solutions of egg-yolk lecithin. In all three cases, the activity was neutralized by specific antibody, and the reaction was not inhibited by the addition of neutralized 0·01 M-potassium hydrogen oxalate.

Table 1. *Titres of a* bifermentans *lecithinase concentrate* (*BL*) *at different* pH *values*

Yolk extract in M/15-acetate-barbiturate buffer as substrate.

pH	BL titre (units/ml.)	pH	BL titre (units/ml.)
3·6	2	6·3	50
4·2	5	7·3	12
5·2	40	8·1	3

Yolk extract. Turbidity and finally curding were produced at pH 4·0–7·2, with an optimum between 5·0 and 6·4 (Table 1). The yolk extract was stable over a range of pH 3·5–8·5, provided that the molarity of the buffers was 0·1 or less. At this molarity, however, the systems tested (borate, acetate, acetate-barbiturate, phthalate, phosphate, glycine-NaCl) were ineffective in varying degrees in buffering BL and WL concentrates, whose pH lay between 6·0 and 6·5, and to a lesser extent against yolk extract, which was usually at pH 7·0–7·5. For determining pH optima, these reagents were first brought to the desired pH with HCl or NaOH solutions, and diluted in the buffer. In all cases the pH of the mixtures was checked colorimetrically immediately after mixing, and when the reaction was complete. At pH values of over 5·0, curding was accompanied by a shift towards lower values. The buffer effects were largely independent of the buffer salts used; M/15-acetate proved most convenient for general use, and for routine titrations buffer at pH 5·1 was employed.

In the absence of added buffer or in the presence of ineffective buffers like M/15-borate pH 7·0, curds were produced by strong BL concentrates in mixtures whose overall pH was in the region of 7·0–7·5. It is possible that the acidity developing during the initial breakdown of lecithovitellin provided at least a sufficient local concentration of hydrogen ions for the reaction to proceed.

Human serum and the action of agar gels. Unlike WL, BL produced neither turbidity nor curding in human serum at 37°, whether buffered at pH 5·1, 5·9 or 7·2 with acetate buffer, or unbuffered in saline. But when strong BL was held at 37° in cups cut out of unbuffered 4 % agar containing 20 % (v/v) human serum of pH 7·5–8·5, a marked turbidity developed to a depth of several millimetres in the agar round the cup. This striking contrast between the results of a tube and a cup-plate test depends on the presence of agar. A concentrated gel is not required; for example, a fluid mixture containing 20 % (v/v) human serum and 32 BL units/ml. at pH 8·4 remained clear for 24 hr. at 37°, whereas in the presence of 0·15 % agar a marked turbidity developed in 2 hr.; and 0·3 % induced marked curding in the same period. The effect was peculiar to BL. The same concentration of agar did not materially enhance the action of WL on human serum at pH 7·2, presumably because WL at this pH was acting within its optimal range. The gel did not adsorb inhibitory substances from the serum, and the activation was not due to soluble substances present in commercial agar; for the supernatant human serum left after centrifugation of serum-agar gels that had been held at 37° for 3 hr. was as insensitive to BL as untreated human serum. It was possible also to speed up the reaction in yolk extract with agar, but the yolk extract was to a large extent unstable in its presence. We have not investigated the agar effect further; it will probably prove to depend on adsorption of one or more reactants or products of the reaction to the surface of the agar micelles in the gel.

Curding of yolk extract and the development of turbidity in human serum agar are both manifestations of BL activity; for the potencies of several BL preparations titrated against the two indicators ran strictly parallel.

Lecithin. Dr H. J. Rogers compared the BL concentrate containing 1024 units/ml. with a *Cl. welchii* filtrate rich in α-toxin (WL) and reports as follows.

Broth preparations liberate acid-soluble phosphate from egg yolk lecithin. Macfarlane & Knight's (1941) method was used, except that the BL hydrolysis was conducted at pH 6·0 without added Ca^{++} ions. Though hydrolysis by BL did not proceed as rapidly as that by WL, the type of lecithinase breakdown appears to be the same in both preparations (Table 2). The pH optimum of the BL is very broad, ranging from 5·0 to 6·1 with a maximum in the region of 6·0. The amount of phosphate liberated at pH 7·0 was about half that liberated at the optimum pH.

BL is not activated by Ca^{++} ions; the degree of hydrolysis was the same with (*a*) no added Ca^{++}; (*b*) 0·01 M-Ca^{++} added; and (*c*) 0·01 M-neutralized potassium hydrogen oxalate added. WL, on the other hand, was completely inhibited by the addition of the 0·01 M-oxalate.

Table 2. *Liberation of phosphate from lecithin by concentrated filtrates from* Clostridium bifermentans (*BL*) *and* Cl. welchii (*WL*)

	Acid-soluble P in hydrolysate (mg./10 ml.)	
Time	**BL**	**WL**
15 min.	0·053	0·200
30 min.	0·080	0·398
160 min.	0·188	—
330 min.	0·347	—
18 hr.	0·421	1·258

Defining the unit of lecithinase activity as the amount of enzyme which will liberate 0·1 mg. acid-soluble P in a defined mixture in 15 min. at 37°, the WL/BL ratio of activities is much smaller than the ratio of yolk extract titres (Table 3). The relatively greater activity of BL in yolk extract might be due to other enzymes. Proteases, for example, can produce turbidity in yolk extract (E. S. Duthie, personal communication) and the BL proved to contain a protease highly active against egg yolk proteins. But the addition of a filtrate from *Chromobacterium prodigiosum*, highly active in digesting casein, to graded dilutions both of BL and WL, did not increase the yolk-extract titres, though significant proteolysis of the egg-yolk proteins was demonstrable in a control mixture of prodigiosum filtrate and yolk extract. The discrepancy in the lecithin and yolk-extract ratios is more probably attributable to other factors. For example, if the *bifermentans* lecithinase were more stable than the *welchii* lecithinase in high dilution, the yolk extract titres of BL at 18 hr. would be disproportionately high; or different degrees of substrate-enzyme dissociation or of inhibition by end-products of the reaction might account for differences in the rates of appearance of curds in yolk extract after an arbitrarily selected period of hydrolysis at 37°.

Table 3. *The relative activities of concentrated filtrates from* Clostridium bifermentans (*BL*) *and* Cl. welchii (*WL*)

		Preparation		
Units		**BL**	**WL**	**Ratio of activities**
Lecithinase units/ml. (Macfarlane & Knight, 1941)	pH 6·0	0·53	500	943:1
	pH 7·0	*c.* 0·25	*c.* 500	2000:1
Yolk extract units/ml., (Miles & Miles)	pH 5·1 1 hr.	24	8,000	333:1
	pH 5·1 18 hr.	400	12,000	30:1
Mouse M.L.D. (intravenous)		<1	1,200	>1200:1

It may be concluded that the turbidity in egg yolk produced by preparations of a *Cl. bifermentans* filtrate (BL) results from the action of a lecithinase C—

that is, a lecithinase of the same *type* as that formed by *Cl. welchii* but that the 18 hr. yolk extract titres are probably gross overestimates of the enzyme potency of BL, as compared with WL.

Stability. Both glycerinated BL and concentrated, pressure-dialyzed preparations retained their activity against yolk extract for over 6 months at 2°. The enzyme is comparatively heat stable. A preparation containing 128 BL units/ml. lost 50 % of its activity on heating to 100° for 10 min. at pH 6·5.

Moderate degrees of shaking do not affect the enzyme, but it is susceptible to oxidation; a weak solution of BL (8 units/ml.) was not affected by 2 hr. vigorously bubbling N_2, but lost 50 % of its activity on exposure for 2 hr. at room temperature to 1/150 20 vol. H_2O_2.

The following antiseptics were added to BL (32 units/ml.) for 24 hr.: excess of toluene and chloroform, 0·25 % formalin, 0·5 % phenol, and 1/10,000 merthiolate. None had any significant effect on the BL titre of the preparation. BL may be preserved for several months at 2° under toluene without loss of activity.

Haemolysis. BL preparations at pH 7·0 containing 256 units/ml. were tested against 5 % (volume of packed centrifuged cells/vol. saline) human, horse, sheep, rabbit, guinea-pig and mouse red cells. No haemolysis was seen after 2 hr. at 37° and overnight at room temperature, excepting in the rabbit and mouse cells, 50 % of which were lysed by a final concentration of 64 BL units/ml. The lysis was inhibited by *Cl. welchii* α-antitoxin. The haemolysis observed on horse blood agar plates round colonies of *Cl. bifermentans* does not appear to be due to the lecithinase; for not all lecithinase-producing strains are haemolytic, and the haemolytic activity of lecithinase-producers is not affected by strong anti-lecithinase sera.

Toxicity. BL was non-toxic to mice by the intravenous route in doses corresponding to 512 BL units, which are equivalent to about 0·5/lecithinase units of Macfarlane & Knight (1941) and, on this basis, equivalent to 0·5 ml. of *Cl. welchii* toxin at pH 7·0. Doses of 100 BL units in 0·1 ml. produced no more than a transient inflammation on intradermal injection in rabbits and guinea-pigs, whereas the corresponding amount of *Cl. welchii* α-toxin, in terms of Macfarlane & Knight's units, produced a marked inflammatory lesion with central necrosis. The simultaneous injection of 1/50,000 adrenaline to prevent dispersal of the injected material (Mason, 1936) enhanced the skin toxicity of this dose of BL; after 24 hr. lesions were on the average 12 mm. in diameter, slightly inflamed, with a central whitish area 5 mm. in diameter, which became scabbed in 3 days; the inflammation was significantly diminished when the BL was mixed with strong α-antitoxin before injection.

In summary, BL is a lecithinase differing from the WL in rate of hydrolytic action in its pH optimum, independence of activation by Ca^{++} ions, and relative non-toxicity for experimental animals (Table 3). Its activity can be titrated by the development of turbidity in yolk extract or in human serum agar, though the relation of titre to degree of hydrolysis of lecithin differs substantially from that of *Cl. welchii* lecithinase. Like WL, BL is a lecithinase C. Its antigenic relationship to WL is discussed in the following section.

THE IMMUNOLOGICAL RELATION OF *CL. BIFERMENTANS* LECITHINASE AND *CL. WELCHII* α-TOXIN

The anti-BL potency of therapeutic *Cl. welchii* antisera used in Nagler tests is roughly proportional to their α-antitoxin content (Dr Nancy J. Hayward, personal communication). Horse antitoxins also contain antibodies to other antigenic components of *Cl. welchii*, and to other bacilli. These antibodies may have been induced naturally, or as the result of the various inoculations to which the horses are subjected. Consequently in such heterogeneous antisera the only reliable indication that the anti-BL and anti-WL action is due to the same antibody is that the anti-BL:anti-WL ratio of potencies should be constant in a large number of sera from different animals; for it is unlikely that two antibodies would be present in the same relative concentration in the sera of a number of horses.

Source of the antisera

Cl. welchii. In all, twenty-four horse antisera containing *Cl. welchii* α-antitoxin were tested. They were mainly single bleedings, each from a different horse, and most of them were 'native' (i.e. unrefined). The results with antisera refined either by ammonium sulphate precipitation or partial proteolytic digestion or with a few native sera dried and reconstituted, did not differ significantly from those obtained with native sera.

Cl. bifermentans. Rabbits were selected whose sera contained no detectable anti-WL or anti-BL activity. They received a thrice-weekly intraperitoneal injection of 2 ml. of a 7 hr. living culture of *Cl. bifermentans*, containing about 50 BL units. The living culture produced no ill-effects. After two 4-weekly courses weak sera were obtained of which 1 ml. neutralized 40–80 BL units. An attempt to improve the antigenicity of the weak BL preparations by alum-precipitation was unsuccessful. Several months later, the same rabbits received six to nine thrice-weekly injections of a concentrated BL containing 2000 units. Sera harvested 7–10 days after the last injection neutralized 5000 BL units/ml.

Precipitin tests

The strong *Cl. bifermentans* antilecithinase sera reacted strongly with BL preparations, giving a typical zone of specific optimal precipitation in a constant-antibody titration; they failed to precipitate WL. Strong *Cl. welchii* antitoxins precipitated WL, but not BL.

Titration of anti-lecithinase sera

(*a*) *Against growing plate cultures*. Falling dilutions of antisera were incorporated in Petri dishes of Fildes's (1920) medium, containing 4 % agar. The plates, together with a control containing normal serum, were seeded with four strains of *Cl. bifermentans* and of *Cl. welchii*, each in one spot. The end-point was taken as the full neutralization of the Nagler zone round growing colonies after 24 and 48 hr. incubation. Estimation of the *bifermentans* end-points was complicated by the presence of faint unneutralized zones of turbidity. Ten horse sera, whose α-antitoxin content ranged from 0 to 4800 i.u./ml. were compared with normal rabbit serum in this way. Only two strong α-antitoxins neutralized the BL as well as the WL zones, giving anti-BL:anti-WL ratios of 1:16 and 1:64. The method was abandoned, partly because high concentrations of antiserum could not be achieved in the agar, and partly because the amount of lecithinase produced by a growing colony was not a satisfactory standard against which to measure the anti-lecithinase potency of the sera.

(*b*) *Against yolk extract in tubes.* Falling dilutions of antisera in 0·25 ml. were mixed with 2 BL or 2 WL units in 0·25 ml. and held at 37° for 30 min.; 0·25 ml. of M/15-acetate buffer pH 5·2 and of yolk extract were added and the result read after 18 hr. at 37°.

The dilutions of the test sera were made as accurately as possible. Taking the appearance of even slight degrees of turbidity as evidence of lecithinase activity, the neutralization of 2 BL units was far from sharp, rising from 0 to 100 % over a 32-fold range of serum dilutions (see Table 4).

Table 4. *The neutralization of BL and WL in constant proportions by* Clostridium welchii *antitoxin S*1

(t), t, t^{+}, t^{++} = degrees of turbidity, trace to marked opacity; t^{+++} = visible flocculation. C3, C4, etc. = floating curd and its thickness in mm. 18 hr. readings at 37°.

Enzyme preparation	Dilution of α-antitoxin						
	1/20	1/40	1/80	1/160	1/320	1/640	1/1,280	Control
BL 2 units	0	(t)	t	t^{+}	t^{+}	t^{+++}	C3	C4
BL 1 unit	0	0	(t)	t	t	t^{+}	t^{++}	C3
BL ½ unit	0	0	0	(t)	(t)	t	t	C1

	Dilution of α-antitoxin						
	1/500	1/1,000	1/2,000	1/4,000	1/8,000	1/16,000	Control
WL 2 unit	0	0	C3	C6	C6	C6	C6
WL 1 unit	0	0	0	C3	C6	C6	C6
WL ½ unit	0	0	0	0	C3	C4	C4

Nevertheless, defining the end-point as the highest completely inhibiting dilution, repeated titrations with a given serum gave consistent results, and neutralization occurred in constant proportions of lecithinase and antilecithinase, as in Table 4, which records a result typical of six *Cl. welchii* antitoxins. But though with a given serum the results were consistent, the range of the 0–100 % response, and particularly the point within the range at which curding appeared, varied with each serum tested. Thus for antiserum S1 in Table 4, the range was 32-fold (1/40 to 1/12,800) and curds were formed over a twofold range (1/640 and 1/1280). With antiserum S10 (Table 5), the range was also 32-fold (1/80–1/2560), but curding took place over an eightfold range (1/300–1/2560). In contrast the neutralization of 2 WL units was sharp, and the dose responses similar.

With the six antitoxins, the anti-BL:anti-WL ratios lay at random between 1:25 and 1:400 (Table 5 (*a*)) irrespective of the anti-WL potency of the sera, or of their state, i.e. native or refined. Repeated titrations with another preparation of BL gave substantially the same results; but with ten native antitoxins included at the same time (Table 5 (*b*)) the ratios all lay between 1:20 and 1:80.

Table 5. *Tube titrations of* Clostridium welchii *α-antitoxins against* 2 *BL and* 2 *WL units*

Cl. welchii α-antitoxins	Titres, 18 hr. Reciprocal of antiserum dilution: anti-BL	anti-WL	Ratio anti-BL:anti-WL
(*a*) S1	20	1000	1:50
S10	40	2000	1:50
G.W.P. 1100/36	10	4000	1:400
G.W.P. 401	10	4000	1:400
K17	40	1000	1:25
G.G. (Toronto)	10	2000	1:200
(*b*) S11	10	400	1:40
S12	20	400	1:20
S13	20	800	1:40
S14	10	400	1:40
S15	20	800	1:40
S16	20	800	1:40
S17	20	400	1:20
S18	40	800	1:20
S19	10	800	1:80
S20	10	400	1:40

It will be evident that the variations of dose-response in the anti-BL titrations and the difficulty of estimating the 0 % neutralization end-point could produce widely varying ratios, even though the anti-BL and anti-WL effects were due to the same antibody. By analogy with titrations of antitoxins in animals, sharper dose responses should be obtained by using a larger amount of BL in the titrations; and this proved to be the case with strong antisera. The method was however inapplicable to all the sera because the anti-BL titres of the sera were so low that neutralization of concentrated BL would have taken place only in neat or 1/2 serum; i.e. dilutions in which non-specific interference with curding of yolk extract may occur. In the hope of obtaining sharper end-points with stronger BL, and yet avoiding non-specific inhibition, we devised a cup-plate method of titration.

(*c*) *Against human serum in cup-plates.* Mixtures of equal volumes of antiserum dilutions and WL or BL were held at 37° for 30 min. Holes 7 mm. in diameter were punched in plates of 4 % agar in unbuffered saline containing 20 % of a strongly Nagler-reacting human serum; these were filled with 0·06 ml. of the reacting mixtures. After 20 hr. at 37°, the end-point was taken as the smallest dilution of serum completely neutralizing the zone of turbidity round the cup.

Yolk extract can replace human serum; but serum is preferable, since the agar is initially more transparent. In human serum agar, the non-specific inhibition observed in tube reactions with native, undiluted normal horse or rabbit sera did not take place, or was minimal; the width of the turbidity zone was sometimes diminished by 0·5 mm., but its intensity was equal to that round the control cup.

As in the tube titrations, for a given antiserum the proportions of lecithinase and anti-lecithinase in just neutral mixtures was constant for an eightfold range of lecithinase concentration. Good zones, 3–5 mm. in width, were produced by preparations containing 8 WL units or 32 BL units/ml. With the proportionally larger dose of BL, the neutralizing end-points were much sharper, and the estimated anti-BL potency of the sera greater than those obtained by tube titrations. The anti-WL potency measured in the cup-plates was slightly higher. For these reasons, and because different amounts of BL and WL were used, the cup-plate potency ratios are not directly comparable with the tube potency ratios.

Twenty *welchii* antitoxins, including the sixteen listed in Table 5, were titrated against equal volumes of WL and BL, 16 and 64 units/ml. respectively. The distribution of anti-BL:anti-WL ratios is shown in Table 6. Having regard to the fact that the titres were obtained with twofold dilutions of antitoxins, the similarity of ratio for all the sera tested provides substantial evidence for the neutralization of the two lecithinases by one antibody.

Table 6. *Cup-plate titrations of twenty* Clostridium welchii *α-antitoxins against BL and WL: the distribution of anti-BL:anti-WL ratios*

Ratio anti-BL:anti-WL	No. of antitoxins
1:2·5	2
1:4	3
1:5	9
1:10	6

In cup-plate titrations, the anti-BL:anti-WL ratios of four strong rabbit *Cl. bifermentans* anti-lecithinase sera were 8:1, 10:1, 16:1 and 16:1. Since all the rabbits were free of antilecithinase before immunization, the fact that the antisera neutralize both lecithinases establishes that the α-toxin of *Cl. welchii* is related to some antigenic constituent of *Cl. bifermentans*, and the similarity of the ratios suggests that the constituent is its lecithinase.

The antitoxic properties of Clostridium bifermentans *antilecithinase*

The most forceful proof of the antigenic relation of BL and WL lies in the fact that bifermentans antilecithinase sera neutralize the necrotic and lethal action of *Cl. welchii* α-toxin and that immunization with BL confers an active immunity to the α-toxin. Two *Cl. welchii* toxins, one a glycerinated concentrate (290), the other a dried toxin precipitated from filtrates by ammonium sulphate (Welchpool) were used. Table 7 summarizes the passive protection tests in 18–22 g. mice. Equal volumes of toxins and antisera were held at room temperature 3–4 hr., and injected intravenously in 0·4 ml. doses. Neat anti-*bifermentans* serum 2737 neutralized about 4 LD_{50} of the Welchpool toxin, and diluted serum was proportionally active. Two other anti-*bifermentans* sera, 2717 and 2736, neutralized toxin 290, and their effect was proportional to their anti-BL titres, which were in the ratio 1:2. Two normal rabbit sera, and four other control rabbit sera, antisera to *Proteus vulgaris* and *Brucella melitensis*, were not antitoxic. In view of the consistently sharp dose response of mice to these preparations of α-toxin (D. G. Evans, personal communication) these results are significant.

Table 7. *The protective action of* Clostridium bifermentans *antilecithinase against* Cl. welchii *α-toxin, injected intravenously in mice*

		Rabbit sera			
		Bifermentans antilecithinase 2737 diluted			Normal
Toxin	Dose of toxin	1/1	1/2	1/4	1/1
		No. of mice dying/group			
Cl. welchii (Welchpool)	1·500 mg.	3/3	3/3	3/3	3/3
	0·750 mg.	0/3	3/3	3/3	3/3
	0·375 mg.	0/3	0/3	1/3	3/3
	0·187 mg.	0/3	0/3	0/3	2/3
	0·093 mg.	0/3	0/3	0/3	0/3

		Rabbit sera		
		2717 1/1	2736 1/1	Normal 1/1
		No. of mice dying/group		
Cl. welchii (290); dilution of glycerol concentrate	1/20	3/3	2/3	—
	1/40	2/3	0/3	—
	1/80	0/3	0/3	3/3
	1/160	—	—	0/3
	1/320	—	—	0/3

The *bifermentans* antilecithinase sera 2717 and 2718 and normal rabbit serum were each mixed with equal volumes of graded dilutions of the toxin (290) and, after standing at 37° for 3 hr., 0·1 ml. of the mixtures were injected intradermally in four 200 g. guinea-pigs. The average reaction after 24 hr. to 1/100 toxin was an inflammatory area 22 mm. in diameter, with a central whitish necrotic area 5–8 mm. in diameter; and to 1/400 toxin, a slightly inflamed area 10 mm. in diameter. The normal rabbit serum did not modify these reactions significantly, but with both antisera the reactions to a mixture of 1/100 toxin and antilecithinase were equivalent to those of 1/400 toxin alone, indicating a four-fold reduction in toxicity.

Active cross immunity was determined in two of the rabbits, 2717 and 2719, used in the preparation of the *Cl. bifermentans* antisera. They were each given a boosting dose of 1024 BL units intraperitoneally and 3 weeks later, when their sera neutralized respectively 1280 and 320 BL units/ml., they were depilated, and graded amounts of *Cl. welchii* toxin (Welchpool) in 0·2 ml. were injected intradermally. Two normal rabbits of about the same weight were also injected. Using either the overall area of gross oedema and inflammation or the central area of necrosis (Table 8) as a measure of toxicity, it was strikingly evident after both 1 and 2 days that the rabbits immunized with BL were protected against the *Cl. welchii* α-toxin.

Table 8. *The active immunization of rabbits* 2717 *and* 2719 *by* Clostridium bifermentans *lecithinase (BL) against* Cl. welchii *α-toxin. Inhibition of skin necrosis 2 days after intradermal injection of toxin*

Dose of Welchpool toxin (mg.)	Area in sq.mm. of skin necrosis in rabbits			
	2717	2719	Control 1	Control 2
2·000	10	34	205	490
1·000	10	13	173	265
0·500	—	3	105	94
0·250	—	—	93	55
0·125	—	—	64	50
0·063	—	—	39	20
0·031	—	—	—	—
0·016	—	—	—	—

A dried preparation of *Cl. sordellii* toxin, which proved to contain about 33 BL units/mg., was tested to ascertain the independence of its lethal action and lecithinase activity. The toxicity was not affected by normal rabbit serum, *Cl. welchii* α-antitoxin (4000 i.u./ml.) or by a *bifermentans* antilecithinase containing 5000 anti-BL units/ml. (Table 9).

Table 9. *Absence of protective action of* Clostridium bifermentans *antilecithinase* 2717 *and* Cl. welchii *α-antitoxin* $S1$ *against* Cl. sordellii *toxin*

Sordellii toxin (dose in mg.)	Nil	Serum		
		Normal rabbit	Anti-*bifermentans*	Anti-*welchii*
	No. of mice dying/group			
0·020	3/3	3/3	3/3	3/3
0·010	2/3	3/3	2/3	3/3
0·005	0/3	—	—	—

DISCUSSION

The serological evidence that both in *Cl. welchii* α-antitoxins and in *Cl. bifermentans* antilecithinase sera one antibody is responsible for neutralizing the two lecithinases has been presented at some length. It was not practicable with the material at our disposal to perform complete 'mirror' tests with antisera to the two lecithinases prepared *ad hoc*. For the exploration of *Cl. welchii* antisera we had to rely on the less satisfactory method of analysing heterogeneous horse antitoxins, which provides only indirect evidence of antigenic similarity. This proved to be difficult, for with certain sera the tube and cup-plate titrations, and consequently the ratios, were not consistent. The inconsistency lay chiefly in the fluctuation of the anti-BL titres in the tube as compared with those in the cup-plate. In the cup-plate, the substrate is reached only by diffusion of the enzyme-antiserum mixtures into the agar gel; it is probable that free enzyme in such mixtures diffuses more rapidly than enzyme-antibody complexes, whereas in the tube, the substrate is in contact with, and perhaps competes with the antibody for the enzyme during the incubation of the mixtures. As the result of some unpublished experiments on the inhibition of antilecithinase sera by the addition of the yolk extract indicator at varying intervals after mixing lecithinase and antiserum, we concluded that competition occurred both with BL and WL; and in a recent paper Zamecnik & Lipmann (1947) have clearly demonstrated such competition in *Cl. welchii* lecithinase-antilecithinase systems. It is significant that the discrepant titres occurred only in the heterologous system—a *Cl. welchii* antibody and a *Cl. bifermentans* antigen—and may be attributable to wide variations in avidity of the antibody for heterologous antigen; variations which are minimized in the peculiar conditions of the cup-plate. Whatever the explanation of the difference, we have accepted the cup-plate titre as a truer measure of the combining power of the antitoxins because end-points were measurable with greater accuracy and non-specific inhibition of curding by undiluted and moderately diluted sera was minimal. Moreover, the evidence of the more consistent cup-plate ratios accords with the direct evidence that known *bifermentans* antilecithinase neutralized the α-toxin of *Cl. welchii*.

Our hope that such an antibody might throw light on the relation of lecithinase-activity to toxicity in the *Cl. welchii* α-toxin has not been fulfilled. The parallelism of lecithinase and toxicity in preparations of this toxin indicates that the two activities are consistently carried on one particle. If, as Macfarlane & Knight (1941) suggested, the lecithinase confers haemolytic and necrotic powers on the α-toxin, then the *bifermentans* antilecithinase should be antitoxic. But the fact that the *bifermentans* antilecithinase is antitoxic does not prove the identity of toxic and enzymic groups; for antibody combining with the lecithinase groups alone might mask adjacent toxic groups in the α-toxin particle; or the specificity of *bifermentans* antilecithinase for α-toxin may be accidental in that it is determined neither by the lecithinase nor the toxic group on the α-toxin particle, but by another determinant, common to both enzymes.

Only a substantial independence of neutralization by *bifermentans* antilecithinase of the lecithinase activity and the toxicity of the α-toxin would have given positive evidence that the two activities were referable to different groups on the particle.

From the fact that among all the lipids they tested, only lecithin was fully effective in interfering with the combination of *Cl. welchii* lecithinase and α-antitoxin, Zamecnik & Lipmann (1947) concluded that the interference was due, not to a non-specific coating of the enzyme by the lecithin, but to competition of lecithin and antibody for the same region of the enzyme molecule. But the demonstration of such inhibition does not necessarily solve the problem of the identity of the hypothetical enzymic and toxic groups in the molecule, for a steric interference with the toxic group might follow a specific adsorption of lecithin to an adjacent non-toxic enzyme group.

Nevertheless, the properties of the *bifermentans* lecithinase itself throw some doubt on the assumption that lecithinase activity *per se* is sufficient to account for the lethal and necrotic powers of the α-toxin. The *bifermentans* lecithinase, in doses sufficient to liberate as much acid-soluble phosphate from lecithin as 0·5 M.L.D. of α-toxin at pH 7·0, was completely non-toxic on intravenous injection in mice, and only mildly inflammatory in the skin of guinea-pigs. At the pH of the tissues, the *bifermentans* lecithinase, unlike α-toxin, is working well outside its optimal pH range though, as the *in vitro* activation of the lecithinase at non-optimal pH values by an agar gel demonstrates, the overall pH condition may be irrelevant provided that certain physico-chemical conditions are satisfied. We have no means of deciding whether these conditions are realized in the tissues; and, moreover, the argument can be applied equally well to the α-toxin of *Cl. welchii*. But the possibility of such activation suggests that the difference between a toxic and a non-toxic lecithinase of the same apparent enzyme type may reside either in a readiness of absorption to certain tissue structures that is independent of the actual lecithinase activity, or in the presence in the body of activators to one but not the other lecithinase.

These speculations can be fully tested only when we have prepared a *bifermentans* lecithinase whose phosphate-liberating activity is equivalent to that of at least 10 M.L.D. of *Cl. welchii* α-toxin.

The similarity of the pH optima suggests that the curding of egg yolk extract and the liberation of phosphate from lecithin are manifestations of the same enzymic process; and the neutralization of both by *Cl. welchii* α-antitoxin, which is unlikely to contain antibodies to two different enzymes of *Cl. bifermentans*, suggests that a protease does not contribute to the curding. The greater sensitivity of the yolk extract to *bifermentans* lecithinase measured in terms of phosphate-liberation, compared with its sensitivity to *welchii* lecithinase, is most probably due to the greater stability of the *bifermentans* lecithinase in the enzyme-substrate mixture; for the ratio of yolk extract titres in a given preparation at 1 hr. is of the same order as the ratio of phosphate-liberating power (Table 3) and the discrepancy develops only after many hours' incubation.

The two lecithinases are too dissimilar in their rates of action on the yolk extract and the various lecithinase preparations were too impure to permit any speculation about the relative amount of enzyme protein which they contained. Hence it is not possible to translate the ratio of anti-BL:anti-WL potencies into a measure of the degree of antigenic relationships of the two lecithinases. But the fact that BL and WL precipitate strongly with their respective homologous antisera, and that cross-precipitation is absent or feeble, suggests that the relation is fairly remote.

The conclusions with regard to *Cl. bifermentans* apply equally to *Cl. sordellii* and *Cl. centrosporogenes*. As far as our experience goes, we agree with Clark & Hall (1937) and Stewart (1938) that these two bacilli are probably identical with *Cl. bifermentans*, and that *Cl. sordellii* is a toxigenic variety of the species. Their similarity is further emphasized by their production of Nagler-reacting substances all of which are neutralized by α-antitoxin (Hayward, 1943) and by the antilecithinase of *Cl. bifermentans*. Both Clark & Hall and Stewart record slight neutralization of the *sordellii* toxin by *Cl. bifermentans* antisera. If *Cl. sordellii* is a variety of *Cl. bifermentans* we may expect that some strains of *Cl. bifermentans* will elaborate the *sordellii* toxin in amounts so small as to be detectable only by the specific antibody response in an animal after a course of immunization; and in these instances the immunizing suspensions used may well have been prepared from a strain of this kind. Antisera to our strain M58*e* had no such antitoxic effect, and neutralization tests showed quite clearly the independence of the *Cl. sordellii* lecithinase and toxin.

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PROCEEDINGS OF THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its **Fourth General Meeting** *in the London School of Hygiene and Tropical Medicine, Keppel Street, London, W.C.* 1 *on Friday and Saturday,* 20 *and* 21 *December* 1946. *There was a Symposium on 'The Nature of Virulence' on the* 20*th at* 10.00 *a.m. and Demonstrations on the* 21*st. The following communications were given during the Symposium:*

Virulence of Bacteria in Plants. By Mrs Dagny Oxford

The bacterial phytopathogens, with occasional exceptions, belong to small sections of the two genera *Bacterium* and *Pseudomonas*, as compared with the great variety of types occurring as animal pathogens. They are free-living, heterotrophic, gram-negative, non-sporing micro-organisms which for the most part grow on the simplest nutrient substrates and are very insusceptible to attack by chemical agents. The fact that the known phytopathogens should be confined to such adaptable, resistant bacteria indicates that the plant host is by its gross anatomy and physiology much more efficient in withstanding bacterial infection than is the animal. An explanation may be given by the following facts: (1) the almost impervious nature of the external surfaces of the plant, (2) the widespread occurrence of phenolic products of metabolism, (3) the less ready availability of peptides and proteins, the bulk of the tissues consisting of very resistant carbohydrate, (4) the lack of a swiftly moving circulatory system, (5) the generally acid reaction of the sap, prevailing aerobic conditions, and subjection to fluctuating temperatures that favour the development of fungi rather than bacteria.

It is generally accepted that bacterial pathogens invade plant tissues mainly by means of wounds, insect bites, abrasions, etc. Considerable progress has been made in elucidating the various nutritional deficiencies or disproportions which weaken the vitality of the plant and, therefore, predispose it to infection. As regards proneness to bacterial infection, however, especially in the case of the leaf-spot and soft-rot diseases, the most important environmental factor known is that of humidity (Ark & Thomas, 1946). Ability to invade the host is only one aspect of virulence. Communicability, the power of becoming established in the host under natural conditions (Coburn, 1944) is a vital part of the problem which in the case of bacterial phytopathogens has not yet received due attention. For obvious reasons, by far the greatest volume of work has been done in connexion with the crown-gall organism (*Bact. tumefaciens*), e.g. Boivin *et al.* (1935) have extracted from the bacterial cells a complex capable of inducing galls, and Riker *et al.* (1945) have made detailed biochemical studies on the products of metabolism of the bacteria.

No detailed comprehensive study has yet been made of the antigenic structure of the flagellate coliform-like organisms described under the generic name of *Erwinia* (Bergey), nor of the pseudomonads separated into the two groups—*Xanthomonas* (Dowson) and *Phytomonas* (Bergey). Their nutrilite requirements have only recently been studied (Starr, 1946). These are the bacteria which break down tissues, cause necroses, wilts and cankers. The means whereby they effect this is unknown. Advance might well be made by a study of their pectolytic enzymes. Many pathogenic pseudomonads produce a levan from sucrose (Cooper & Preston, 1935), and this phenomenon might be utilized protectively. In the case of *Pseudomonas mors-prunorum*, the agent of bacterial canker of plums, a cell-free filtrate has been obtained which induces lesions similar to those caused by the bacterium (Erikson & Montgomery, 1945). A mass of data on the host-range of these pathogens is available (Wormald, 1933). It is most desirable that the information to be gained from such lines of approach should be collated, and strained analogies with certain aspects of bacterial disease in animals be avoided in order to obtain a true picture of the nature of virulence in plants.

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Virulence of Bacteria in Animals. By D. W. Henderson

The term 'virulent' is properly used in the sense of being poisonous. Over the years since the microbial theory of infection was established the term has had impounded into it the innumerable variables that are associated with both host and parasite in the initiation of an infectious disease. It is understood that the discussion is meant to embrace problems associated with such host parasite relationships rather than with the more limited problem of capacity to poison. It may be that this interpretation of what the Society would wish to discuss is wrong. In that eventuality guidance offered in general discussion will be welcome to many of us who until now have used the term 'virulence' in all shades of meaning.

It is proposed to introduce for discussion by brief illustration such problems as the capacity of an organism to poison the host directly or indirectly, to invade, to remain within the host for long periods apparently in symbiosis, to join forces with a newcomer, to adapt to environment and to change habit according to the method of meeting the host. In addition it would seem proper to discuss the variability of the host in relation to physical environment as well as strain and species differences. This wide field appears essential since parasite action cannot be discussed without reference to host response.

Among the organisms I propose to mention in illustration will be *B. typhosum*, *Str. pneumoniae*, *B. anthracis*, *B. prodigiosum*, *Cl. welchii*, *Cl. botulinum*, *Cl. tetani*, *Cl. septique*, *C. diphtheriae*, *H. influenzae*.

Virulence in Protozoa. By H. E. Shortt

In dealing with the concept 'The Nature of Virulence', which is the subject of this symposium, an attempt is first made to define the term 'virulence', more especially in relation to the Protozoa. One immediately comes up against difficulties, even in framing a definition. Terms such as 'invasiveness' and 'pathogenicity' at once come to mind but one soon realizes that neither of these attributes of any organism is synonymous with virulence, although both are essential to the production of virulence. The attempt at definition of the term 'virulence' is further complicated by realization of the essential role played, both by the nature and extent of the defence mechanisms of the invaded host.

This is equivalent to saying that there is no such character as intrinsic virulence in any protozoan and that the term 'virulence' must be considered only as the resultant of a complex of interacting factors. It is obvious that before this interaction can be brought about there must be a very close association between parasite and host and, therefore, the various methods by which such associations can be brought about and maintained is considered and illustrative examples are given of each of these methods. Having thus cleared the ground and considered the various factors involved in the production of virulence in Protozoa an attempt is then made to define the concept 'virulence in Protozoa'. Three essential factors are considered necessary to the production of virulence in the case of any protozoan. These are:

(*a*) The capacity to multiply in the tissues of the host.

(*b*) Actual invasion of the tissues of the host.

(*c*) The power to overcome the defence mechanisms of the host sufficiently to produce disease in the latter, disease being understood to imply either actual direct destruction of tissues or gross impairment of function due to whatever cause may be operative in individual cases.

These three factors are considered in turn individually and illustrative examples are given under each heading from the various classes of the Protozoa, practically all of which supply examples of virulence under the appropriate conditions.

Finally a few questions bearing on the general theme, as developed in the preceding sections, are posed for consideration and discussion. These are:

(1) Does a virulent parasite, in the sense I have defined virulence, i.e. producing an obvious diseased condition, inherently change its character and become non-virulent when the condition it has caused becomes chronic or latent and inapparent, e.g. latent malaria, in either mammalian or avian hosts?

(2) Is the virulence complex an advantage or a disadvantage to a parasite?

(3) Does virulence represent the evolutionary end-result of invasion of a host by a parasite or does it represent the initial stages of association between the two, finally to be succeeded by commensalism or symbiosis?

Virulence of Fungi in Plants. By E. Gäumann

Virulence of Fungi in Animals. By J. T. Duncan

Some of the dermatophytes, on their specific host, offer perfect examples of efficient parasitism in which the host-parasite equilibrium may remain undisturbed for years, while the host even affords the parasite protection and aids the distribution of its spores. During epidemics of this kind of ringworm infection the host-parasite balance may tend to be disturbed, and the lesions become less simple in character; to the disadvantage of the parasite. These epidemics are sometimes attributed to an increased virulence of the parasite, ignoring the more plausible explanation—diminished resistance of the host. Such, apparently, increased infectivity of the fungus may, therefore, be relative. A similar explanation may be applied to epidemics of thrush amongst infants in crèches, although the infectivity of the causative fungus, *Candida albicans*, is known to be exalted by passage in a series of animals. An alien ringworm fungus on a particular host may be more pathogenic but less infective than the species naturally parasitic on that animal.

Some of the deeper mycoses are caused by fungi which vegetate normally as saprophytes and are only occasionally pathogenic. Infection depends upon the mechanical introduction of the fungus into the tissues through penetrating wounds or open lesions of the skin, and the simultaneous introduction of an inert foreign body on which the fungus may have been living, may be a necessary factor. Fungi of this kind may not be capable of extreme morphological modification, and in adaptation to parasitic life they merely form a compact colony in which the mycelium is usually arranged in radial fashion, with thick-walled cells, like chlamydospores, in the peripheral part. In this kind of infection there is no tendency to metastatic spread, and the fungus shows no invasive power beyond a limited centrifugal extension of the colony. New colonies may, however, arise in the neighbourhood of the primary colony and by repetition of this process a whole member, such as a foot, may become involved, but the spread of the disease seems to be due to the action of wandering phagocytes which pick up portions of the living fungus and transport them to new sites in which new colonies develop. Thus the disease remains localized and a condition of stalemate exists between parasite and host in which the infection may endure for many years. In the case of these fungi it is usually found impossible to infect laboratory animals either with material from the parasitic colony or cultures isolated from it. Fungi of this group, although pathogenic, would seem to have no property of virulence.

In a more important group of systemic mycoses the infecting fungi possess the property of dimorphism; vegetating in normal saprophytic life in a more or less complex mycelial form, but capable of developing, for parasitic life in animal tissues, a simple form, often unicellular and yeast-like, adapted for rapid reproduction and invasion. Fungi of this group may be highly infective and invasive, often causing generalized and fatal disease. Although transmission of the disease from man to man, directly, is almost unknown, except in rare post-mortem room 'accidents', the saprophytic form of the fungus is virulent in a high degree and this virulence seems to be related to the property of dimorphism. In culture at 37° C., on certain substrata, the dimorphic character can be demonstrated in saprophytic life.

With the pathogenic fungi, therefore, virulence (interpreted in the restricted sense of infectivity and invasive power) would seem to be largely a matter of adaptability, morphological and physiological, to parasitic life in animal tissues.

Virulence of Viruses in Plants. By Kenneth M. Smith

The truth of the matter is that we have very little idea of what virulence in a plant virus consists and it is, in consequence, a difficult matter to discuss. It may be well, therefore, in the first place to attempt to give a definition of what is meant by the term 'virulence'. It is true to say that the highest point of virulence is reached when the death of the host ensues and since the cell and not the whole organism is the host of the virus, any virus which kills the cells but not necessarily the whole plant may be said to be *virulent.* By this definition, then, is virulence ever an intrinsic property of a virus or is it merely a host-parasite relationship? It is possible to find facts which support to a certain extent both these hypotheses. First, if virulence is an intrinsic property of a virus, there should exist viruses which kill the cells of all host-plants under all environmental conditions. Obviously, however, intolerance on the part of the host if carried to this extent would involve extinction of the virus. Intensely necrotic or virulent viruses of this type would need an extremely efficient method of spread if they were going to survive. This is so for the following reasons: viruses which kill the cells produce only necrotic symptoms which may take the form of a lethal systemic necrosis or else localized necrotic spots in which the virus is imprisoned and cannot spread farther through its host. In either case such a virus is to all practical purposes non-infectious since it cannot spread by contact and no insect vector is likely to feed on patches of dead cells. Indeed plants which react in this manner are said to be 'field immune' and any virus which produced such symptoms on all hosts would rapidly be lost. A virus of this type might perhaps be defined as intrinsically virulent, but do such viruses exist?

It is possible to arrange viruses in a graded series of virulence starting with one which most nearly approaches our definition of intrinsic virulence and gradually descending the scale to the virus which is carried without symptoms by a large number of plants. Indeed paradoxically enough the virus, which most nearly fulfils our definition of intrinsic virulence, is also a virus which can be carried without symptoms by many host plants and thus completes the circle. Tobacco necrosis virus is virulent to all plant species to which it has been experimentally transmitted and becomes systemic in none. Such a virus would be rapidly lost were it not for one fact. In nature this virus occurs only in the roots of plants where it produces no visible reaction and it is the change of location from the roots to the aerial portions of the plant which develops the virulence.

In discussing virulence as a virus-host relationship it is easy to quote examples of viruses virulent on one host plant and avirulent or even symptomless on others. The classic instance is the behaviour of the ordinary type strain of tobacco mosaic virus on tobacco and *Nicotiana glutinosa*, mottling and distortion in the former, death of the cells in the latter. Again, tobacco ringspot virus causes necrotic and chlorotic rings on the tobacco plant but kills *Datura stramonium* and *Phaseolus vulgaris* outright. That these reactions are due to a relationship between host and parasite and not to an intrinsically virulent virus can easily be demonstrated by inoculating from an infected plant on which the virus is virulent to a plant on which the virus is avirulent and mild symptoms in the latter will always result.

It is possible to alter experimentally the intrinsic virulence of a plant virus and also to alter the virus-host relationship so that virulence of a virus to a particular host is temporarily increased. Thus, a strain of tobacco mosaic virus has been isolated after irradiation which produces a lethal or severe necrosis of tobacco plants instead of the familiar mottling. While it cannot be said definitely that this mutation was due to the irradiation the fact of the increased virulence cannot be denied. There are various references in the literature to apparent increases of virulence in viruses by passage of particular hosts or merely by continuous passage of susceptible hosts. The best known case is the apparent reduction in virulence of curly-top virus by passage of the weed *Chenopodium murale* and its restoration to original virulence by passage of *Stellaria media*. This phenomenon, however, can also be explained on the basis of a *selection* of strains rather than an increase in virulence. Thus it can be assumed that there are present two strains, a mild and a severe one, and in *Chenopodium murale* the mild strain outgrows the severe one whilst in *Stellaria media* the reverse is true, although in both cases some of each strain remains.

As regards virulence as a virus-host relationship, this can be increased in various ways. Growing the plants under conditions of high temperature and light intensity induces tobacco mosaic virus to kill *Nicotiana glutinosa* with a systemic necrosis. Site or method of entry of the virus may also influence virulence. Thus transmission of curly-top to tomatoes by grafting with scions from infected tobacco plants which have recovered from the symptoms induces a mild disease but tomato plants infected by leafhoppers feeding on the outside leaves induces a severe disease. Similarly *N. glutinosa* grafted with a scion of mosaic tobacco dies with systemic necrosis but if inoculated with the same virus develops only local lesions.

Virulence of Viruses in Animals. By C. H. ANDREWES

Though viruses are apparently simpler than bacteria, Protozoa and fungi, the problem of their virulence seems just as complex, if not more so. It is proposed to limit the term to consideration of properties of the virus which determine the issue of the host-virus struggle, once a virus-particle has made contact with a potential host. [Whether a strain of virus causes a lethal epidemic may depend on other properties of the virus, e.g. on how effectively virus is dispersed from one infected host and on how readily it travels (because it resists desiccation or is effectively conveyed by a vector and so on).] Virulence, as a phenomenon in the field, depends on the ability of a virus particle which has reached its host to pass the superficial defences, enter, multiply and produce disease or death. To the laboratory worker a virulent virus is often thought of as one with a small L.D.$_{50}$, under specified conditions; but that definition depends on over-simplification.

Viruses are believed to be intracellular parasites. Most evidence suggests that phagocytes and opsonins are relatively unimportant in defence. In the simplest conception, the ideal, perfectly virulent virus gets unimpeded to a susceptible cell, multiplies therein with great rapidity, disrupts the cell and is effectively conveyed to more susceptible cells, the result being maximum damage to the host. It is, however, uncertain whether mere rapidity of mass-production of vast numbers of virus particles is enough to make a virus virulent. Direct destruction of the infected cells must be important. There is also the question of whether intact virus particles in large enough quantity may not exert some toxin-like effect, e.g. rickettsiae, influenza. Possibly also the presence of dead or less perfectly adapted virus particles may slow down the whole process (interference phenomenon).

Amongst the neurotropic viruses we find great differences in ability to reach the C.N.S. from the periphery. Tissue culture may change the properties of a virus so that it can no longer pass to the C.N.S. and kill, though its L.D.$_{50}$ after intracerebral injection is unaltered. Other factors affecting virulence:

(i) *Aptitude for synergism with secondarily invading bacteria.* 'Virulent smallpox' is often smallpox complicated by streptococcal infection. It has been suggested that the agent of 1918 pandemic 'flu differed from ordinary 'flu in the greater tendency of the virus to associate itself with haemophilus and with pathogenic cocci.

(ii) *Affinity for certain tissues or organs.* Attack of poliomyelitis on the medulla makes the disease more lethal and this may either depend on an intrinsic property of the virus, or on something more or less accidental.

(iii) *Change in antigenic constitution.* Where a community has a certain level of resistance to a virus—as to influenza, a mutation affecting the antigenic structure of the virus may render it capable of attacking people immune to ordinary strains: this is a likely explanation, at least in part, of the events of the 1918 pandemic.

Finally, a common feature of viruses is that adaptation to a particular host by passage in it increases virulence for that host with, as a rule, concomitant decrease in resistance for other hosts, e.g. influenza in ferrets and mice.